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Time: 9:00 a.m.

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DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
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John Sarvey, Ph.D. Department of Pharmacology Major Advisor	✓	
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James Columbe, Ph.D. Department of Anatomy and Cell Biology Member	✓	
Aviva Symes, Ph.D. Department of Pharmacologoy Member	✓	

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"Activation of Mitogen-Activated Protein Kinases and Cyclic AMP Response Element-Binding Protein in Synaptic Plasticity"

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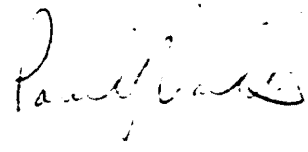

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ABSTRACT

Title of dissertation: Activation of Mitogen-Activated Protein Kinases and Cyclic AMP Response Element-Binding Protein in Synaptic Plasticity

Pamela J. Voulalas, Doctor of Philosophy, 1997

Dissertation directed by: John M. Sarvey, Ph.D., Professor, Department of Pharmacology

Current evidence supports a critical role for cAMP in synaptic plasticity. Forskolin increases adenylyl cyclase activity to generate cAMP which induces a long-lasting potentiation of excitatory postsynaptic potentials in the hippocampal dentate gyrus to 137 ± 5% of control, which persists for at least 60 min after forskolin removal. Blockade of NMDA (N-methyl-D-aspartate) receptors with 20 μM 2-amino-5-phosphonovalerate (APV) or L-type calcium channels with 10 μM nifedipine, reduced this potentiation. TMB-8 (8-(diethylamino)octyl-3,4,5-trimethoxybenzoate, 50 μM), which interferes with calcium release from an inositol-3,4,5-trisphosphate (IP_3) receptor-sensitive internal pool, also reduced forskolin potentiation. These data indicate that calcium from both extracellular and intracellular pools

mediate the long-lasting, cAMP-mediated potentiation induced with forskolin in the dentate gyrus.

The transcription factor CREB (cAMP response element-binding protein) and the mitogen-activated protein kinases (MAPKs) are transducers of membrane-to-nucleus signaling that are activated by phosphorylation. Fifteen to 20 min after application, forskolin stimulated phosphorylation of CREB and both 42kD and 44kD MAPK. The tyrosine kinase inhibitors, lavendustin A (5μM), methyl 2,5-dihydroxycinnamate (MDC, 20μM), and the MAPK kinase (MEK) inhibitor PD098059 (50μM), blocked CREB phosphorylation and also reduced synaptic potentiation. PD098059, but not MDC, blocked MAPK phosphorylation. NMDA receptor blockade reduced forskolin potentiation, but did not prevent forskolin-induced MAPK phosphorylation. Inhibition of IP_3-regulated calcium release with TMB-8 depressed forskolin-induced potentiation and prevented CREB phosphorylation, but did not block increases in MAPK phosphorylation. These distinctive effects probably reflect input from different biochemical pathways during potentiation.

Long-term potentiation was induced with high-frequency stimulation in dentate gyrus of anesthetized rats and in dentate slices. Increases in CREB phosphorylation were NMDA receptor-dependent. CREB phosphorylation was preceded by increases in MAPK phosphorylation, which were not NMDA receptor-dependent. LTP *in vitro* was blocked by PD098059. These data demonstrate sequential and differential

activation of CREB and MAPKs during LTP, and suggest a link between NMDA receptor activation and CREB phosphorylation.

ACTIVATION OF
MITOGEN-ACTIVATED PROTEIN KINASES
AND
CYCLIC AMP RESPONSE ELEMENT-BINDING PROTEIN
IN SYNAPTIC PLASTICITY

by

Pamela Joi Voulalas

Dissertation submitted to the Faculty of the
Department of Pharmacology Graduate Program of the
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DEDICATION

To my mother, Carol Hood Voulalas, whose primary ambition was to instill within me strong feelings of independence and capability.

ACKNOWLEDGEMENT

I gratefully acknowledge my mentor, Dr. John Sarvey. A true *maestro*, he has been patient, supportive, upbeat and knowlegeable throughout the course of this work. I have tremendous respect for his expertise, and appreciate the respect he has always demonstrated for me as together we worked through the details of this project.

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understand better the micro-climate of a brain slice incubator in diverse environments.

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LIST OF ABBREVIATIONS

AMPA.....Amino-3-hydroxy-5-methyl-4 isoxazole propionic acid
ANOVA.....Analysis of variance
APV.....2-Amino-5-phosphonovalerate
ATP.....Adenosine triphosphate
BCA.....Bicicconinic acid
BDNF.....Brain-derived neurotrophic factor
BSA.....Bovine serum albumin
cAMP.....Cyclic adenosine-3',5'- monophosphate
CICR.....Calcium-induced calcium release
CREB.....cAMP response element-binding protein
DMSO.....Dimethyl sulfoxide
EPSC.....Excitatory postsynaptic current
EPSP.....Excitatory postsynaptic potential
ERK.....Extracellular signal-regulated kinase
GABA.....Gamma-aminobutyric acid
HFS.....High-frequency stimulation
HRP.....Horseradish peroxidase
IBMX.....Isobutylmethyl xanthine
ISOP.....Isoproterenol
IICR.....Inositol-1,4,5-trisphosphate receptor-induced calcium release
i.p. .....intraperitoneal
IP_3.....Inositol-3,4,5-trisphosphate
IPI.....Interpulse interval
kD.....kiloDaltons
kHz.....kiloHertz
LFS.....Low-frequency stimulation
LTP.....Long-term potentiation
MDC.....Methyl 2,5-dihydroxycinnamate
MAPK.....Mitogen-activated protein kinase
MEK.....MAPK and ERK kinase
mg.....Milligram
mGluR.....metabotropic glutamate receptor

MK-801....an NMDA receptor antagonist
mRNA.....messenger RNA
ms.....Millisecond
mV.....Millivolt
NE.....Norepinephrine
NELLP.....Norepinephrine-induced long-lasting potentiation
NGF.....Nerve growth factor
NMDA.....N-methyl-D-aspartate
NT-3.....Neurotrophin 3
PD 98059..MEK inhibitor
PMSF.....Phenylmethylsulfonyl fluoride
PTP.....Post-tetanic potentiation
SDS.....sodium dodecylsulphate
SER.....smooth endoplasmic reticulum
STP.....short-term potentiation
TBS.....Tris-buffered saline
TK.....Tyrosine kinase
TMB-8.....8-(diethylamino)octyl-3,4,5-trimethoxybenzoate

INTRODUCTION

BACKGROUND

Over the past hundred years research in the field of learning and memory has focused on the contribution of different structures in the central nervous system to the acquisition, retention and recall of information. Early behavioral evidence implicated the hippocampus as critical to certain forms of learning and memory (Olton, 1986). For example, therapeutic destruction of the temporal lobe for the suppression of seizures in epileptic humans produced both anterograde and retrograde amnesia (Milner, 1972). Comprehensive examination of the effects of such lesions in humans, combined with comparable studies in laboratory animals led researchers to conclude that the hippocampus plays an important role in laying down intricate neuronal networks for information storage and retrieval. Early studies in animals employed specific behavioral tasks to assess hippocampal function after lesioning, or while under the influence of a pharmacological agent. Tasks that involve the use of environmental spatial cues to obtain reward (Olton et al., 1979; Morris, 1984), and tasks that result in context-dependent fear conditioning (Kim and Fanselow, 1992;

Phillips and LeDoux, 1992) are examples of tests of hippocampal function.

The results obtained by researchers in the field of learning and memory ultimately dictated that we assess mnemonic processes at the molecular level. Amino acids, cations and short-lived gaseous molecules have been identified as extracellular triggers for the cascades of intracellular events believed to underlie synaptic plasticity. Through the early 1980's, basic electrophysiological and pharmacological approaches were the primary tools used by researchers to study hippocampal function. Currently, researchers are expanding their use of technical approaches in order to understand the molecular mechanisms underlying synaptic plasticity in the hippocampus. Biochemical and molecular biological techniques are extensively utilized to achieve this goal. This area of research is now at the point where expression of specific genes can be manipulated in order to understand their role in hippocampal function, from the behavioral level to the level of a single neuron.

LONG-TERM POTENTIATION: A CELLULAR MODEL FOR LEARNING

As behaviorists and neurologists provided further support for the hippocampus as a fundamental structure in

memory, so continued the studies by electrophysiologists to dissect the neuronal pathways and to map the circuitry of this highly laminar structure first described by Ramón y Cajal in 1909 (Andersen et al., 1971; Andersen, 1975). The 'tri-synaptic' circuit begins with perforant path fibers entering from the entorhinal cortex and synapsing on dentate granule cells (see Fig. 1). Axons from the granule cells (known as mossy fibers) form synapses on field CA3 pyramidal cells, which themselves project to the field CA1 pyramidal cells via the Schaffer collateral system (Ramón y Cajal, 1909; Andersen, 1975). In these three fields, the evoked responses recorded extracellularly are population evoked potentials (population spike, PS) and excitatory postsynaptic potentials (EPSP). The PS is recorded in the cell body layer, and represents the summation of synchronous firing of action potentials (Andersen et al., 1971). The EPSP of the medial perforant path is recorded in the dendrites comprising the middle third of the molecular layer of the dentate gyrus. Its characteristic negative-going potential represents the influx of cations into the dendritic area at the recording site during depolarization of the postsynaptic cell (Dahl and Sarvey, 1989).

With the progression of these electrophysiological studies came the discovery that if presynaptic inputs to a neuron were repetitively stimulated with a high frequency train of current pulses, a sustained increase in activity occurred in the postsynaptic neuron. This phenomenon was

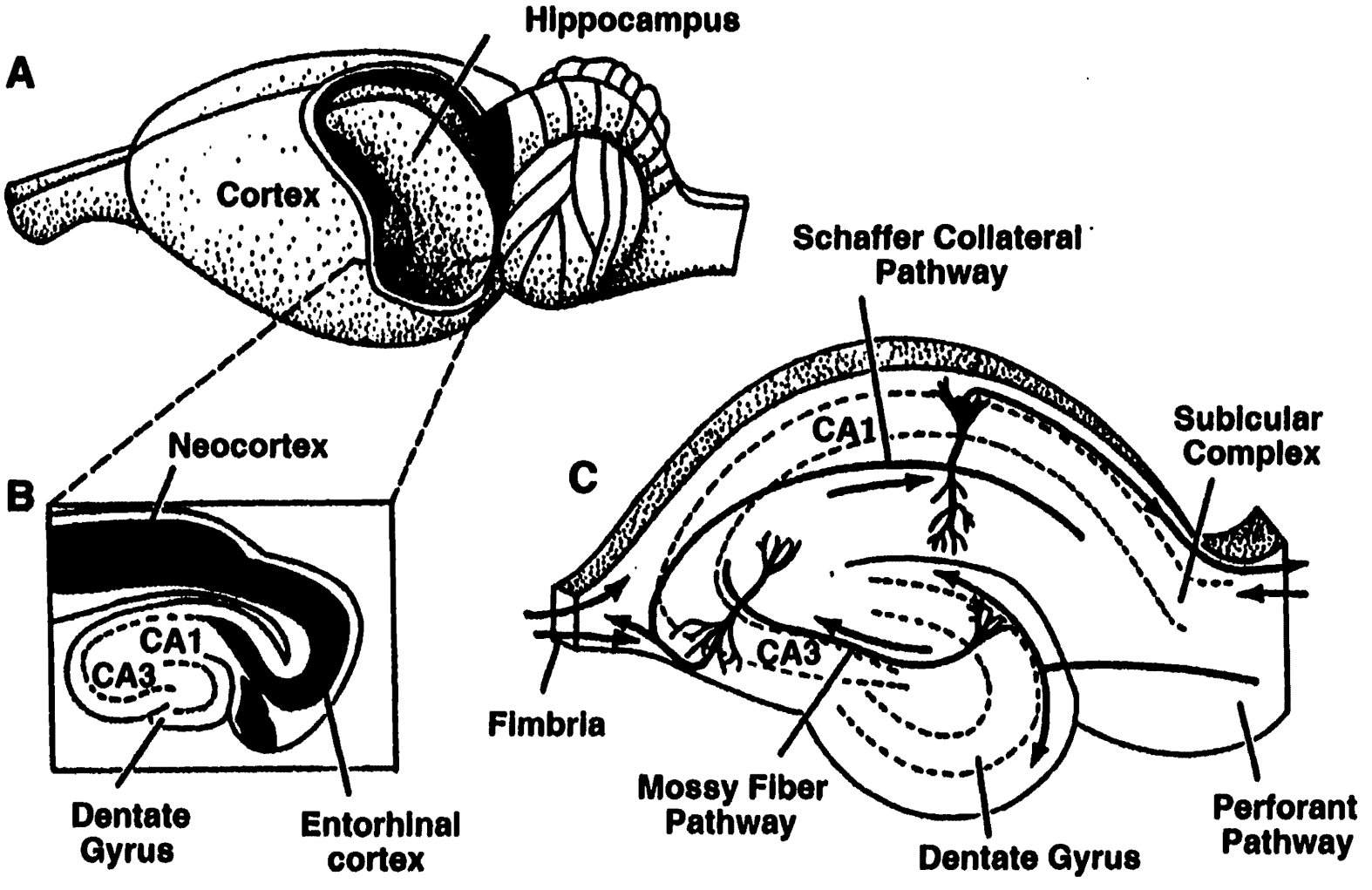


Fig. 1. The rat hippocampus. **A**, orientation of the right and left hippocampi within the brain. **B**, representation of a horizontal section through the brain, showing association of the hippocampus with the entorhinal cortex and neocortex. **C**, an enlarged and detailed view of the transverse slice of the hippocampus as it is used for electrophysiological studies, showing the three main fields: CA1, CA3 and dentate gyrus. Neuronal cell bodies are in densely packed layers as indicated by the dashed lines. (After *An Introduction to Molecular Neurobiology*, 1992, p. 485, Zach W. Hall, ed., Sinauer Associates, Sunderland, MA.)

originally observed in rabbits (Bliss and Lømo, 1973; Bliss and Gardner-Medwin, 1973) and was described as "a long-lasting potentiation" of neuronal responses. We now call this long-term potentiation (LTP), and it has been observed in all three hippocampal fields. LTP has since been elicited in rabbits, guinea pigs, and rats *in vivo* using awake, freely moving animals and anesthetized animals.

Development of the *in vitro* slice preparation (Gibson and McIlwain, 1965; Yamamoto and McIlwain, 1966) and its application to the hippocampus greatly facilitated the study of LTP at these synapses. While lacking the intricate network of an intact hippocampus, this preparation allowed for better control of electrode placement and more precise determination of concentration of agents used in pharmacological studies. Although the *in vivo* model retains all of the normal modulatory inputs missing in the slice preparation, the slice represents a more stable preparation, being freed of the respiratory, circulatory or anesthetic effects inherent in the *in vivo* preparation.

LTP in the hippocampus has been characterized in the following way. First, there appear to be three different phases associated with LTP. The first characteristic response to high-frequency stimulation (HFS) is post-tetanic potentiation (PTP). This is an immediate and dramatic increase in the amplitude of the response, but is of short duration. Within seconds to minutes this maximal response has decreased, though the magnitude is still elevated over

baseline. A second phase was postulated and referred to as short-term potentiation, or STP, which appears to last on the order of minutes to tens of minutes (Stanton and Sarvey, 1984; Scharfman and Sarvey, 1985). Expression of STP is dependent upon activation of NMDA receptors in field CA1 and the dentate gyrus (Collingridge, Kehl and McLennan, 1983; Wigström et al., 1986) Finally, the duration of LTP was the premier characteristic that led to the idea that it might in fact be a good cellular representation of learning and memory. Potentiated responses were shown to remain elevated for hours in the slice and for days to weeks in the animal (Bliss and Lømo 1973; Bliss and Gardner-Medwin 1973; Douglas and Goddard 1975).

MECHANISMS OF SYNAPTIC PLASTICITY IN THE DENTATE GYRUS

The descriptive terms "induction" and "maintenance" refer approximately to PTP/STP and LTP, respectively. A more definitive description of the different phases is emerging as investigators pharmacologically and genetically dissect LTP and determine which second messengers and proteins affect one, all, or none of these three phases (Rosahl et al., 1993).

In the hippocampal dentate gyrus and CA1, experimental results obtained primarily with the in vitro

slice preparation led to the conclusion that two events required for induction of LTP were (1) presynaptic activity coupled with (2) postsynaptic depolarization. Presynaptic release of glutamate activates postsynaptic (AMPA) receptors which mediate sodium and potassium fluxes through the postsynaptic membrane. This produces a depolarization in the postsynaptic neuron sufficient to relieve a magnesium block on the NMDA receptor, which, in the presence of a co-agonist glycine, activates the postsynaptic, calcium-passing receptor. Such pre- and postsynaptically coupled activity satisfies the requirements for a hypothesis put forth by Donald Hebb in 1949. He proposed that "the persistence or repetition of a reverberatory activity (or 'trace') tends to induce lasting cellular changes that add to its stability." His hypothesis states "If an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency as one of the cells firing B is increased" (Hebb, 1949). Current thought is that Hebb's postulated "metabolic change" is mediated by increases in intracellular calcium concentration. Calcium and cAMP mediate changes in cellular activity ranging from phosphorylation to changes in gene expression. Mounting evidence indicates that such events define the mechanisms underlying short- and long-term phases of LTP.

In our own laboratory we have targeted our analyses toward the molecular mechanisms of LTP, beginning with the

thesis project of Patric Stanton. He provided the first demonstration that protein synthesis was required for LTP (Stanton and Sarvey, 1984). He also showed that depletion of norepinephrine (NE) by 6-OH dopamine lesion or blockade of β-adrenergic receptors led to the inability to elicit LTP in the dentate gyrus, thus defining a critical role for NE and cAMP in LTP (Stanton and Sarvey, 1985b, 1987). Finally, he was able to demonstrate that bath application of a β-receptor agonist to hippocampal slices resulted in a lasting potentiation in the dentate gyrus, that did not require any electrical stimulation but did require NMDA receptor activation (Burgard et al., 1989; Stanton and Sarvey, 1985a, 1987).

Calcium and LTP

Calcium influx in the presynaptic terminal following depolarization and action potential firing in neurons is essential for neurotransmitter release (Katz and Miledi 1967). Numerous experiments have also demonstrated that postsynaptic calcium is required for expression of LTP (Barrionuevo and Lynch, 1983; Malenka et al., 1988). The primary mediator of calcium influx in postsynaptic neurons is the NMDA receptor, and in field CA1 and the dentate gyrus activation of this receptor is required for induction of LTP (Collingridge et al., 1983; Harris et al., 1984; Wigström et

al., 1986; Abraham and Mason, 1988; Perkel et al., 1993). Activation of NMDA receptors is also required for norepinephrine-induced long-lasting potentiation (NELLP) in the dentate gyrus (Burgard et al., 1989).

Although initial experiments demonstrated an absolute requirement for activation of NMDA receptors in field CA1 and the dentate gyrus, forms of LTP that are non-NMDA receptor-dependent were subsequently described in fields CA1 and CA3. In field CA1, voltage-sensitive calcium channels appear to mediate potentiation induced with a high-frequency train, while blocking NMDA receptors with the specific antagonist D-APV (Grover and Teyler, 1990). In field CA3, LTP of the mossy fiber pathway is also non-NMDA receptor dependent, invoking L-type calcium channels for its induction (Harris and Cotman, 1986; Weisskopf et al., 1994).

Cyclic AMP and LTP

Cyclic AMP is generated from ATP by adenylyl cyclase, which itself is stimulated by G protein-linked receptors such as β-adrenergic receptors. Norepinephrine acting at these receptors modulates cAMP levels in the brain (Woodward et al., 1979). Early studies describing a role for norepinephrine in the dentate gyrus resulted from experiments in which application of norepinephrine produced a long-lasting increase in the amplitude of the dentate granule cell

population spike (Neuman and Harley, 1983; Hopkins and Johnston, 1984). Further evidence supporting NE as a modulator of LTP included studies demonstrating that rats depleted of NE by 6-OH dopamine lesion were deficient in LTP in slices from the dentate gyrus. Elevation of cAMP with a low concentration of forskolin in slices from lesioned rats rescued LTP in the NE-depleted tissue (Stanton and Sarvey, 1985b, 1987). The characteristic effects of NE administration were blocked by β-receptor antagonists. These data provided support for the idea that the effects of NE in the dentate were mediated by activation of a β-adrenergic receptor linked to a G-protein which stimulated adenylyl cyclase to generate cAMP (Stanton and Sarvey, 1985b, 1985a).

Glutamate is the primary excitatory neurotransmitter in the hippocampus. Lynch and Bliss (1986) assayed for stimulation of release of glutamate from hippocampal synaptosomes by α- and β - adrenergic receptor agonists and antagonists. They demonstrated that significant amounts of glutamate were released from dentate slices when β-adrenergic receptors were bound by the agonist isoproterenol, or α_2-adrenergic receptors were bound by the agonist clonidine. They therefore concluded that glutamate release by NE was from presynaptic elements in the dentate gyrus.

To substantiate this finding, another group pressure-ejected NE onto hippocampal slices from guinea pig and observed a resultant increase in the peak inward calcium current in granule cells (Gray and Johnston, 1987). NE

increased the duration and amplitude of calcium spikes and this effect was reproduced by application of forskolin and 8-Bromo-cAMP (a non-hydrolyzable analog of cAMP). An interesting follow-up to this work was a study done in field CA3 in rats, looking at the effect of bath application of isoproterenol on calcium channel activity (Fisher and Johnston, 1990). Using the cell attached patch clamp technique, they inferred from the conductances measured that both N- and L-type calcium channels were being activated. An important observation was that the channels under the patch pipette were being stimulated by isoproterenol to open, though the channels never came into direct contact with the ISOP in the bath. The conclusion was that β-adrenergic receptor activation was transmitting some signal intracellularly.

NE may elicit its effects by stimulating glutamate release, but data supporting a specific action of NE at the presynaptic terminal are not conclusive. However, other studies have also supported a presynaptic role for NE. Treatment of dentate slices from young rats with either NE or ISOP increased the phosphorylation of the synapsins I, IIa and IIb. When phosphorylated, these proteins facilitate transmitter release via their interaction with synaptic vesicles. Aged rats showed no increase in phosphorylation of these proteins, which investigators proposed to be the defect underlying their deficits in LTP (Parfitt et al., 1991). This effect of isoproterenol on synapsin phosphorylation was

found only in the dentate gyrus, and not field CA1 (Parfitt et al, 1992). In field CA1, application of forskolin together with the phosphodiesterase inhibitor IBMX was found to enhance both spontaneous and evoked release of transmitter (Chavez-Noriega and Stevens, 1994).

Data in support of a postsynaptic role for NE in modulating synaptic changes come from more recent phosphorylation studies carried out on cloned glutamate receptors located on the postsynaptic membrane. Transfection of cloned GluR6 (kainate subtype of non-NMDA glutamate receptor) in human kidney cells mediated current flow in response to glutamate and this effect was potentiated by either cotransfection of protein kinase A (PKA), addition of PKA or cAMP to the pipette, or bath application of forskolin (Raymond et al. 1993; Wang et al. 1993).

To conclude this section, mention needs to be made of one important characteristic of LTP which is shared by NELLP. In the dentate gyrus both LTP and NELLP require activation of the NMDA receptor for induction of potentiation. Burgard et al. (1989) demonstrated that the NMDA receptor antagonists APV and CPP both elicit a concentration-dependent block of both processes. The dependence of forskolin-induced long-lasting potentiation on NMDA receptors is less restrictive, in that NMDA receptor blockade yields only a reduction in the full expression of potentiation by forskolin (Voulalas and Sarvey, 1997b).

Tyrosine kinases and LTP

Tyrosine kinases (TK) are a class of cellular enzymes recognized as important modulators of cell growth and differentiation. TK activity is typically associated with membrane-bound growth factor receptors, but cellular homologues of oncogenic proteins such as *fyn, yes, abl* and *src* also possess TK activity. TKs were first identified in transformed cells where the levels of enzyme activity and tyrosine phosphoproteins was extremely high, but tissue-specific and subcellular localization of TK activity has subsequently been detected in normal tissue as well (Swarup et al., 1983; Hirano et al., 1988; Pang et al., 1988; Ellis et al., 1988). These sites include the brain, which is among those normal, nonproliferating tissues with the highest TK activity. TK activity in the brain is associated with the activation of a number of growth factor receptors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin (Nairn et al., 1985). TK activity is also associated with the Trk receptors A, B and C, which are activated by neurotrophic factors specifically localized to neuronal tissue.

The hippocampus was one of the brain regions in the rat central nervous system found to possess the highest

endogenous TK activity (Hirano et al. 1988). Subcellular fractionation of rat forebrain demonstrated that synaptic vesicles retained the greatest amount of TK activity (Hirano et al. 1988; Pang et al. 1988; Ellis et al. 1988). This finding was of great importance to the study of synaptic plasticity, as a postulated function for tyrosine phosphoproteins such as growth factor receptors in a non-proliferative cell type was to mediate structural changes in the synapse. That is, acquisition of novel information is thought to culminate in the growth or modification of a synapse.

The role of neurotrophins and their receptors in plasticity was first addressed in single cell preparations. TK activity has since been implicated in the induction, and perhaps even the maintenance of plasticity in the hippocampus. One of the first studies correlating activation of a tyrosine kinase with LTP demonstrated that bath application of EGF and fibroblast growth factor (FGF) to rat hippocampal slices produced an enhancement of the population spike (PS) in CA1 pyramidal cells, but no change in the excitatory postsynaptic potential (EPSP) (Terlau and Seifert, 1989; Seifert and Terlau, 1990). In support of these findings, LTP has been effectively blocked with TK inhibitors in field CA1 *in vitro* (O'Dell et al., 1991) and dentate gyrus *in vivo* (Abe and Saito, 1993). Finally, a direct action of neurotrophic factors on hippocampal responses has been documented, albeit with conflicting outcomes. One group

described a brief bath application of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) that gave rise to a potentiation which persisted after washout of the neurotrophins, and was NMDA receptor-dependent (Kang and Schuman, 1995). Another group reported that the neurotrophins merely increased the ability to elicit LTP in field CA1 in a developmentally-specific manner (Figurov et al., 1996). It appeared that TK activity, thus far known to be mediated by growth factors through their receptors, could have significant effects in a terminally differentiated cell type (neuron). Could these TK activities be involved in the growth of a synapse when potentiated? Downstream targets of Trk receptor activation include the mitogen-activated protein kinases (MAPKs).

The MAPKs, also known as extracellular signal-regulated kinases (ERKs) have recently emerged as important cytosolic signal-transducing molecules that are activated by phosphorylation via a dual-specificity kinase. MAPK kinase (now known as MAPK/ERK kinase or MEK) was the first kinase shown to phosphorylate a substrate (MAPK) on both a threonine and tyrosine residue (Seger and Krebs, 1995). A study in cultured hippocampal neurons provided one of the first reports of a link between activation of NMDA receptors by glutamate and calcium-dependent stimulation of phosphorylation of MAPK on tyrosine residues. Bading and Greenberg (1991) demonstrated that treatment with glutamate

or NMDA resulted in an increase in phosphorylation of MAPK that was blocked by APV.

The first demonstration that TKs were involved in LTP was in field CA1, showing blockade of LTP with TK inhibitors (O'Dell et al., 1991). The inhibitors were only effective if applied prior to induction of LTP, implying a role for TK activity in the induction of LTP. Grant et al. (1992) examined knock-out mutants for four non-receptor TKs found in the hippocampus: *fyn, yes, abl* and *src*. They found only the *fyn*$^-$ mutants deficient in LTP and a spatial learning task. A major problem with the interpretation of the results was the obvious developmental defects in the *fyn*$^-$ mouse noted in histological stains of these hippocampi. Additionally, some *fyn*$^-$ mutants were still able to exhibit LTP. The lack of a demonstrable effect of the other three kinases does not necessarily indicate absence of function for these kinases in the hippocampus. One explanation is that the roles these particular kinases play is duplicated by other cytoplasmic kinases.

Protein synthesis and LTP

Before the phenomenon of LTP had been described, several investigators had noted inhibitory effects of protein synthesis inhibitors on hippocampal function and memory (Cohen et al., 1966; Barondes, 1970; Flood et al., 1973). The report of *de novo* synthesis of proteins in the

hippocampal slice after stimulation strengthened the idea that sustained increases in hippocampal neuronal activity involved increased protein synthesis (Duffy et al., 1981). Stanton and Sarvey (1984) were the first to demonstrate that LTP was blocked by protein synthesis inhibitors *in vitro*. As a number of inhibitors of protein synthesis have since been shown to block LTP *in vitro* and *in vivo*, it became clear that the long-lasting nature of LTP in the hippocampus is dependent upon protein synthesis (Frey et al., 1988; Otani et al., 1989; Huang et al., 1994). Interestingly, NELLP is also blocked by protein synthesis inhibitors (Stanton and Sarvey, 1985a).

SUMMARY

One of the principal questions for investigators in the field of learning and memory is the following: how is a brief stimulus translated into a long-lasting change in synaptic efficacy?

Because granule cell responses in the dentate gyrus potentiate following either electrical stimulation or chemical elevation of cAMP, I have chosen to characterize "potentiation" in this region. I have focused on the question of whether distinct methods of inducing potentiation all lead to the activation of a similar or distinct intracellular cascade of events. More precisely, does induction of an apparently identical (electrophysiologically) potentiation by electrical or chemical stimulation activate parallel or convergent pathways? To do this I have utilized two stimulus paradigms for inducing potentiation in the dentate gyrus. High-frequency electrical stimulation to induce LTP is known to depend upon calcium for its expression. Chemical stimulation by bath application of forskolin induces a long-lasting potentiation that is mediated by cAMP. The work presented here addresses two important issues. First, are CREB and MAPK activated by phosphorylation in response to the induction of LTP and forskolin-induced potentiation? Second, if both proteins are activated, do they demonstrate similar pharmacological sensitivity? Could these data be used to

deduce whether induction of electrical (LTP) or chemical (forskolin) potentiation activates parallel or convergent intracellular pathways leading to protein synthesis?

Specific Aims

The specific aims of this study were to:

1) characterize pharmacologically forskolin-induced potentiation in the dentate gyrus.

2) determine whether induction of LTP or forskolin potentiation stimulate CREB and MAPK activation, as assessed by changes in their phosphorylation state.

3) examine the sensitivity of changes in CREB and MAPK phosphorylation to pharmacological agents that interfere with the expression of LTP and forskolin potentiation.

This thesis is comprised of four manuscripts which are presented as four sections. A general discussion follows

the manuscripts. To facilitate review of the literature, all references from all papers are presented together at the end of this dissertation.

All of the work presented in this dissertation is original, and all of the experiments were conducted by myself, with the exception of the patch clamp experiments in Part I, and the electrophysiological experiments with PD098059 in Part IV.

Forskolin-Induced Long-Lasting Potentiation in Rat Hippocampal Dentate Gyrus Requires Calcium from Extracellular and Intracellular Sources

SUMMARY

A great deal of evidence supports a critical role for cAMP in synaptic plasticity. Long-term potentiation in hippocampal fields CA1, CA3 and dentate gyrus involves changes in intracellular cAMP concentrations. Using the *in vitro* slice preparation, we analyzed potentiation in rat dentate gyrus induced by elevating intracellular cAMP concentrations. Forskolin directly activates adenylyl cyclase to increase cAMP, giving rise to a long-lasting potentiation, which our data suggest requires calcium for expression. Our aims were to assess the contribution of evoked synaptic activity and to determine the source, or sources, of calcium utilized in the process of forskolin-induced potentiation. Stimulation of presynaptic fibers was not necessary for eliciting potentiation with forskolin. Blockade of NMDA receptors with 20 μM D-APV or L-type calcium channels with 10 μM nifedipine reduced potentiation. TMB-8 (50 μM), which interferes with release of calcium from an IP_3 receptor-sensitive internal pool, also reduced the potentiation induced by forskolin. Heparin in a whole cell patch pipette did not affect potentiation of the EPSC, suggesting a presynaptic role for IP_3 receptors. A role for calcium-induced calcium release was also tested. Pretreatment of slices with 20 μM dantrolene had no effect on potentiation. From these data we conclude that sources of

calcium from both extracellular and intracellular sites mediate the long-lasting, cAMP-mediated potentiation induced with forskolin in the dentate gyrus.

Key words: calcium; dentate gyrus; forskolin; heparin; hippocampus; inositol trisphosphate; learning and memory; long-term potentiation; synaptic plasticity; TMB-8

INTRODUCTION

Cyclic AMP and calcium are two second messengers critical for mediation of long-lasting synaptic changes in the central nervous system. The molecular mechanisms underlying long-term potentiation (LTP), for example, involve pre- and postsynaptic changes in cAMP (Stanton and Sarvey, 1985b; Blitzer et al., 1995; Lopez-Garcia et al., 1996) and calcium (Malenka et al., 1992; Regehr and Tank, 1992; Perkel et al., 1993). Concentrations of these two second messengers are modulated by receptors coupled to G proteins and by voltage and ligand-gated ion channels. Beta-adrenergic receptors linked to G_s elevate cAMP through activation of adenylyl cyclase, and evidence exists that this activity can support potentiation in the dentate gyrus (Neuman and Harley, 1983; Lacaille and Harley, 1985; Stanton and Sarvey, 1985a). Electrical (Collingridge et al., 1983; Grover and Teyler, 1990) and chemical (Burgard et al., 1989) stimulation of neurons can activate NMDA receptors and voltage-gated calcium channels, key mediators of calcium influx from the extracellular environment. Recently described mechanisms for mobilization of calcium from intracellular pools include calcium-induced calcium release (CICR), and inositol trisphosphate (IP_3) receptor-mediated calcium release (IICR) (Henzi and MacDermott, 1992). Activation of the receptors and ion channels functionally linked to increases in cAMP and calcium initiates a cascade of signaling events that is

thought to converge at the level of the nucleus (Sheng et al., 1990; Impey et al., 1996). Subsequent alteration in gene expression leading to variations in synthesis of specific proteins is hypothesized to underlie the physical transduction of a brief stimulus to a persistent alteration in the structure of the synapse (Duffy et al., 1981; Stanton and Sarvey, 1984; Frey et al., 1988; Otani and Abraham, 1989; Geinisman et al., 1996).

We have examined a form of potentiation in the dentate gyrus that is induced by brief bath application of forskolin to hippocampal slices (Sarvey and Voulalas, 1995; Voulalas and Sarvey, 1995). Others have observed potentiating effects of forskolin in fields CA1 and CA3 (Chavez-Noriega and Stevens, 1992; Pockett et al., 1993; Huang et al., 1994). The resulting elevation of intracellular cAMP concentrations gives rise to a lasting change in neuronal activity that we believe must involve calcium fluxes from pharmacologically definable sources. Our aim in this study was to understand induction of potentiation by forskolin. We determined its dependence on presynaptic stimulation, and the chemical basis of its long-lasting nature. We also assessed its requirement for elevation of intracellular calcium. APV was utilized to examine NMDA receptor involvement, and nifedipine was used to determine contribution from L-type calcium channels. In addition, we employed pharmacological agents targeting sources of intracellular calcium release. Our data support recruitment

of both cell membrane-associated and intracellular receptors for calcium flux to elicit potentiation by bath application of forskolin.

MATERIALS AND METHODS

Preparation of hippocampal slices

Male Sprague Dawley rats (Taconic, Germantown, N.Y.) weighing 100-200 g were anesthetized with ketamine hydrochloride (100 mg/kg i.p.) and decapitated. Transverse slices (400 μm) of hippocampus were prepared using a McIlwain tissue chopper. Slices were placed in a modified Oslo interface recording chamber at 32-34° C and perfused at a rate of 3 ml/min with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 1.75, KH_2PO_4 1.25, $MgSO_4$ 1.3, $NaHCO_3$ 26, $CaCl_2$ 2.4, and dextrose 10; pH was adjusted to 7.4 by bubbling with 95% CO_2/5% O_2. Slices were allowed to equilibrate for at least two hours before recordings were initiated.

Electrophysiological recordings

Stimuli were delivered to medial perforant path fibers (middle third of the molecular layer) through a monopolar electrode (75 μm diameter Teflon-insulated stainless steel wire, exposed only at the tip). Extracellular recordings were obtained using standard glass micropipettes filled with 2 M NaCl, 3-8 MΩ resistance. Recording electrodes were positioned a minimum of 500 μm from the stimulating electrodes, and lowered to a final depth of

80 to 120 μm into the slice (Dahl and Sarvey, 1989; Bramham and Sarvey, 1997). Slices were selected for experimentation based upon (1) the absence of secondary population spikes in the evoked response in the dentate granule cell body layer at maximal stimulus intensity (100-150 μA, 200 μs) and (2) the demonstration of paired-pulse depression of the half-maximal population spike at a 20 ms interpulse interval. Only slices showing complete abolition of the second spike were selected for study. Evoked EPSPs (50% of maximum amplitude; 1-2 mV and subthreshold for a reflected spike) were recorded in the mid-molecular layer of the dentate gyrus. Isolation of medial perforant path responses was confirmed by paired-pulse depression seen at an 80 ms interpulse interval using a current intensity which elicited an EPSP that was just subthreshold for a reflected spike (McNaughton, 1980; Colino and Malenka, 1993). Test stimuli were delivered every 30 s. Drug applications were initiated after at least 20 min of stable recording.

EPSCs were recorded from granule cells in whole-cell configuration (Hamill et al., 1981) using a "blind" approach with 3-13 MΩ borosilicate glass pipettes pulled on a Flaming-Brown horizontal puller (Sutter Instruments). Intracellular solution contained (mM) K-gluconate 120, CsCl 10, K-ATP 5, Na-GTP 0.35, sodium phosphocreatine 5, $MgSO_4$ 3, EGTA 0.6, HEPES 3; pH to 7.2 with CsOH; osmolality was adjusted to 300 mOsm/Kg with sucrose. Data were recorded with an Axopatch 1-D amplifier (filtered 2 kHz; digitized 15 kHz) and pCLAMP6

software (Axon Instruments). Holding potential was -60 mV. Heparin sodium salt (200 units/ml) was included in the intracellular solution in some experiments. A 10-min baseline EPSC was recorded before perfusion with forskolin (50 μM for 20 min) was begun. Only cells with seals greater than 1 GΩ, series (access) resistance ≤ 30 MΩ (uncompensated, monitored throughout experiment) and resting membrane potential on breaking in more negative than -70 mV were used. In all experiments, the field EPSP was monitored to verify the efficacy of forskolin.

Drug application

Application of pharmacological agents was achieved by switching the chamber infusion to ACSF containing the drug. Forskolin and its analogs were applied for 20 min; application of inhibitory compounds preceded forskolin by 20 min, and were continued during and for 20 min after the treatment with forskolin; dantrolene was applied for 60 min prior to forskolin application. Experiments with and without antagonists were grouped and compared taking into account the batch of forskolin used and the time-frame of usage. All agents were made up as 1000-fold stock solutions. Forskolin, dantrolene (LC Labs), 1,9-dideoxyforskolin (Calbiochem) and nifedipine (Sigma) were dissolved in dimethyl sulfoxide (DMSO); final concentration of DMSO was no greater than 0.2%. DMSO alone (0.3%) had no effect on basal transmission. D-APV

(Tocris Cookson), 6β-[β'-(piperidino)propionyl]-HCl forskolin (RBI), TMB-8 (LC Labs) and heparin (RBI) were dissolved in water.

Data analysis and statistics

EPSPs and population spikes were amplified, low-pass filtered (up to 3 kHz) digitized at 20 kHz (DAS-20 interface, Keithley Metrabyte, Taunton, MA) and stored for analysis using the Labman data acquisition and analysis program (generous gift of Dr. T. Teyler, NeuroScientific Laboratories, Rootstown, OH). Measures of synaptic efficacy were made using the initial slope of the EPSP. Initial slope measured after 60 min washout of forskolin was normalized to percentage of the mean baseline value.

Data are expressed as the mean ± SEM. Statistical analysis of drug and forskolin effects were carried out in Statview (Abacus Concepts, Berkeley, CA) using the paired t-test, Student's t-test or ANOVA plus a post hoc Bonferroni/Dunn test for multiple comparisons. A probability of 0.05 was selected as the level of statistical significance.

RESULTS

Effects of forskolin and its analogs

Figure 1 provides an illustration of the time course of potentiation induced in the dentate gyrus by forskolin. In these slices (Fig. 1, $n = 5$), bath application of 50 μM forskolin resulted in an increase in the initial slope of the EPSP recorded in the mid-molecular region within 20 min after forskolin addition (130 ± 5% of baseline, $P < 0.05$, paired t-test), which was sustained for 60 min of washout (137 ± 5%, $P < 0.05$). The potentiation led to an increase in the amplitude of the population spike as well (Fig. 1A). Perfusion of slices with 25 μM forskolin also produced a long-lasting elevation of the EPSP slope over baseline levels (60 min wash, 127 ± 11%, $P < 0.05$, $n = 8$). Since we were conducting a parallel biochemical analysis using 50 μM forskolin and wanted to be above threshold for inducing potentiation, we chose 50 μM forskolin for this study. Use of 100 μM forskolin produced a significant potentiation (60 min wash, 146 ± 9%, $P < 0.05$, $n = 6$) which was not different from potentiation induced by 50 μM forskolin ($P > 0.05$, ANOVA, *post hoc* Bonferroni/Dunn test). The potentiation has been noted to last more than 3 h after washout of forskolin with ACSF (data not shown). In control experiments, perfusion of slices with 0.3% DMSO vehicle alone for 20 min

produced no effect (60 min wash, 95 ± 13%, $P > 0.05$, $n = 3$, paired t-test).

The potentiating effect of forskolin is mediated by direct activation of adenylyl cyclase. As shown in Figure 1*B*, perfusion of slices with 50 μM 1,9-dideoxyforskolin (DDF), an analog of forskolin which lacks the ability to activate adenylyl cyclase, not only did not yield any potentiation, but actually decreased the EPSP (60 min wash, 87 ± 6%, $P > 0.05$, $n = 3$, paired t-test).

The argument could be made that the long-lasting nature of the forskolin-induced potentiation is due to an inability to wash out the forskolin. In Figure 2 we show that 50 μM 6β-[β'-(piperidino)propionyl]-HCl forskolin, a water-soluble form of forskolin, (Laurenza et al., 1987) also resulted in a long-lasting potentiation of the EPSP slope (60 min wash, 139 ± 5%, $P < 0.05$, $n = 4$, paired t-test). These values were comparable in magnitude to that elicited by the DMSO-soluble forskolin ($P > 0.05$, ANOVA, with Bonferroni correction).

We tested whether evoked release of transmitter during application of forskolin was required for long-lasting expression of potentiation. Figure 3 shows that interrupting test stimulation during the application of 50 μM forskolin had no effect on potentiation of the EPSP slope when stimulation was reinstated (forskolin 20 min, 143 ± 4%) or after 60 min of wash (133 ± 6%, $P < 0.05$, $n = 5$, paired t-test). The degree of potentiation observed in the absence of

presynaptic stimulation was not different from potentiation induced with either the DMSO- or water-soluble forms of forskolin ($P > 0.05$, ANOVA, plus *post hoc* Bonferroni/Dunn test).

Effect of APV and nifedipine on forskolin potentiation

We tested the hypothesis that a rise in intracellular calcium levels modulates the potentiation induced with forskolin. This could be achieved by calcium entering the cell from extracellular sources via ligand-gated or voltage-gated calcium channels and/or by release of calcium from internal pools. To assess the involvement of NMDA receptors and L-type calcium channels we used D-APV and nifedipine.

Activation of NMDA receptors is a hallmark for the expression of LTP in the dentate gyrus and field CA1. We investigated their involvement by treating slices with 20 μM D-APV, a concentration that blocks LTP and norepinephrine-induced long-lasting potentiation in the dentate gyrus (Burgard et al., 1989). APV was perfused for 20 min prior to introduction of 50 μM forskolin, and was continued for 20 min into the washout. Figure 4*A* shows the effect of blockade of NMDA receptors with D-APV on forskolin-induced potentiation. The initial slope of the EPSP was significantly different from baseline after washout of forskolin (60 min wash, 120 ± 7%, $P < 0.05$, $n = 7$, paired t-test). These slices demonstrated a significant difference in the EPSP slope

compared to slices treated with forskolin alone ($P < 0.05$, ANOVA followed by Bonferroni correction), indicating that D-APV treatment acted to reduce, but not block potentiation induced with forskolin.

We also asked whether L-type calcium channel activation could act as a critical source of calcium influx into the postsynaptic neuron. For these experiments we bracketed forskolin application with 10 μM nifedipine, a concentration that significantly reduced calcium channel-dependent LTP in CA1 (Grover and Teyler, 1990). These results are presented in Fig. 4*A*. Nifedipine reduced (117 ± 6%, $P < 0.05$, $n = 8$, ANOVA, Bonferroni correction) but did not block ($P < 0.05$, paired t-test) potentiation 60 minutes after washout of forskolin.

Simultaneous blockade of both NMDA receptors with D-APV and L-type calcium channels with nifedipine also resulted in a reduction of potentiation when compared to slices treated with forskolin alone (126 ± 6%, $P < 0.05$, $n = 8$, ANOVA with Bonferroni/Dunn *post hoc* test). In summary, NMDA receptors and L-type calcium channels contribute to an influx of calcium that appears to affect the full expression of forskolin-induced potentiation.

Role of IP_3 receptors in forskolin potentiation

The agent 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) interferes with the process of

calcium release mediated by IP_3 receptors via a mechanism not completely understood (Palade et al., 1989). Slices were treated with TMB-8 for 20 min prior to forskolin perfusion. TMB-8 was continued during and for 20 min following forskolin application. Neither 1 nor 10 μM had any effect on potentiation (data not shown). In Fig. 4*B*, we show that 20 μM TMB-8 reduced potentiation (128 ± 5%, $P < 0.05$, $n = 8$, ANOVA, Bonferroni correction) and a concentration of 50μM TMB-8 also reduced potentiation after 60 min of wash (118 ± 6%, $P < 0.05$, $n = 4$, ANOVA, Bonferroni correction). Very high concentrations of TMB-8 (100 μM) completely blocked potentiation, but also decreased transmission with extended exposure to the drug (data not shown).

Figure 4*C* summarizes the results displayed in Fig. 4*A* and 4*B*. In this series of experiments, various treatments were compared to slices treated with forskolin alone, with forskolin producing a potentiation of 165 ± 15% ($n = 5$) after 60 min of wash. These data suggest that the potentiation induced with forskolin is dependent on calcium.

With bath application of TMB-8, we could not be certain whether the inhibitory effects of the drug were presynaptic, postsynaptic or both. To explore a possible postsynaptic role of IP_3 receptors, we made whole cell patch clamp recordings from dentate granule cells stimulated with forskolin and included heparin (200 U/ml) in the patch pipette. Heparin is an IP_3 receptor inhibitor that is not membrane-permeable, and a concentration of 200 units/ml has

previously been shown to suppress the metabotropic glutamate receptor response in CA1 pyramidal neurons (Shirasaki et al., 1994). In cells perfused with K-gluconate buffer alone, the EPSC amplitude was potentiated to 156 ± 23% after one hour washout of forskolin (n = 10), which was not different from potentiation in cells perfused with heparin (219 ± 33%, n = 6, $P > 0.05$, Student's t-test). Forskolin-induced long-lasting potentiation could be recorded in cells from both groups for up to three hours. The absence of an inhibitory effect of heparin on potentiation implies that activation of postsynaptic IP_3 receptors is not being evoked by forskolin. In addition, one can only infer from these data that the effect of TMB-8 is presynaptic.

Role of calcium-induced calcium release in forskolin potentiation

The mechanism of neuronal calcium-induced calcium release from various intracellular sites has been found by others to be regulated by receptors sensitive to dantrolene (for review see Henzi and MacDermott, 1992). We tested the effect of 20 μM dantrolene pretreatment on forskolin-induced potentiation (Fig. 5A). This concentration of dantrolene perfused for 60 min prior to stimulation of slices has been found to be effective in inhibiting physiological responses in the hippocampus. An example is the neuroprotective action of dantrolene on dentate granule cells after injury

(amputation of dendrites) (Soltesz and Mody, 1995). However, this 60 min pretreatment (continued while forskolin was applied) did not block potentiation induced by forskolin (60 min wash, 138 ± 8%, $P < 0.05$, $n = 4$, paired t-test).

DISCUSSION

We sought to understand the mechanism whereby forskolin potentiates responses in the dentate gyrus following elevation of cAMP in hippocampal neurons. We confirmed the long-lasting effects of forskolin on the EPSP recorded in the dendritic region and the population spike recorded in the granule cell body layer. A water-soluble forskolin analog produced an equivalent effect. The inactive compound dideoxyforskolin consistently reduced the initial slope of the EPSP by decreasing a potassium channel conductance (Hoshi et al., 1988; Harris-Warrick, 1989). We established that presynaptic stimulation is not required for expression of potentiation. Forskolin-induced potentiation is reduced by the NMDA receptor antagonist D-APV and the L-type calcium channel blocker nifedipine. Inhibitory effects on potentiation were seen with TMB-8, which interferes with IP_3-mediated calcium release from intracellular pools. A role for calcium-induced calcium release in forskolin potentiation was not supported in this study, as dantrolene did not inhibit expression of potentiation by forskolin. Considering all of these data, we propose that NMDA receptor, L-type calcium channel and IP_3 receptor involvement in the potentiation induced with forskolin reflect a dependency of this phenomenon on calcium from both extracellular and intracellular pools.

Evoked release of transmitter is not required for forskolin potentiation

We saw long-lasting potentiation in the absence of stimulation of presynaptic medial perforant path fibers during forskolin application. When stimulation was resumed, potentiation was as great as in slices that were stimulated at 0.033 Hz throughout forskolin application. Potentiation was maintained at this level throughout 60 min washout of forskolin. It was demonstrated previously that long-lasting potentiation of medial perforant path-granule cell EPSPs by specific activation of adenylyl cyclase linked to β-adrenergic receptors does not require electrical stimulation (Dahl and Sarvey, 1990).

Evidence from several laboratories supports both presynaptic and postsynaptic roles for cAMP in mediating long-lasting changes in synaptic efficacy in the hippocampus. Cyclase activation presynaptically could activate the transmitter release mechanism and facilitate transmitter release (Parfitt et al., 1991; Parfitt et al., 1992; Chavez-Noriega and Stevens, 1994), while postsynaptically located cyclases could enhance responsiveness of glutamate receptors to transmitter (Wang et al., 1991; Greengard et al., 1991; Gean et al., 1992; Raymond et al., 1993; Wang et al., 1993; Benke et al., 1996; Raman et al., 1996). Alternatively, both pre- and postsynaptic effects of adenylyl cyclase activation

could contribute to a long-lasting alteration in the activity of the postsynaptic neuron.

Forskolin potentiation involves extracellular calcium

LTP in the dentate gyrus and field CA1 is NMDA receptor-dependent (Collingridge et al. 1983; Wigström et al. 1986). Norepinephrine-induced long-lasting potentiation (NELLP) in the medial perforant path of the dentate gyrus is produced by bath application of norepinephrine or isoproterenol to slices (Stanton and Sarvey, 1985c; Dahl and Sarvey, 1989). This treatment elevates cAMP (Stanton and Sarvey, 1985b) to yield a potentiation that is also NMDA receptor-dependent (Burgard et al., 1989; Dahl and Sarvey, 1990). In contrast to LTP and NELLP, APV produced only a significant reduction of forskolin potentiation. This outcome was unexpected, in view of the sensitivity of NELLP to APV in the dentate, but in field CA3 forskolin-induced potentiation was also shown to be NMDA receptor-independent (Huang et al., 1994). As will be discussed below, cooperativity may exist between NMDA receptor-mediated influx of calcium and receptors mediating release of calcium from internal sites (Markram and Segal, 1992).

When L-type calcium channels were blocked, forskolin potentiation was reduced compared to that seen in slices perfused with forskolin alone. In other hippocampal regions, other forms of LTP do not require NMDA receptors. For

example, in field CA3, mossy fiber LTP does not depend on NMDA receptors for induction, but may recruit calcium channels (such as the L-type calcium channels) to elevate intracellular calcium (Jaffe and Johnston, 1990). In field CA1, an L-type calcium channel-dependent form of LTP that is independent of NMDA receptor activation has been described (Grover and Teyler, 1990). Considering that the NMDA receptor and L-type calcium channels are important regulators of calcium influx from the extracellular milieu during the induction of LTP, it was curious that pharmacological blockade of both these receptors did not completely eliminate potentiation by forskolin. Thus, forskolin-induced potentiation must involve either a calcium-independent mechanism or regulation of calcium release from critical intracellular pools.

Calcium from intracellular stores regulates potentiation

Intracellular storage sites for calcium are usually, but not exclusively, found in smooth endoplasmic reticulum (SER). In neurons, SER is found in the soma, axons, dendrites and dendritic spines (Henzi and MacDermott, 1992). IP_3 receptors mediate release of calcium from SER stores. In our slices, expression of forskolin potentiation was reduced in a concentration-dependent manner by bath-application of TMB-8, implicating involvement of IP_3 receptors in this phenomenon. The lack of effect on potentiation with

intracellular heparin suggests a presynaptic locus for TMB-8-sensitive IP_3 receptors. Autoradiographic localization of IP_3 receptor binding has revealed receptors in the molecular layer of the rat dentate gyrus (Worley et al., 1989). IP_3 receptor immunoreactivity has been noted in presynaptic terminals and dendritic spines of rat cerebellar Purkinje cells (Henzi and MacDermott, 1992). Forskolin's ability to affect calcium fluxes is supported by studies demonstrating phosphorylation of the IP_3 receptor by PKA (Supattapone et al., 1988; Yamamoto et al., 1989), which facilitates release of calcium from that store when IP_3 is bound (Volpe and Alderson-Lang, 1990; Nakade et al., 1994). With regard to plasticity in the hippocampus, activation of mGluR linked to IP_3 production enhances NMDA receptor-mediated currents (O'Connor et al., 1994) giving rise to persistent, long-lasting calcium current oscillations in CA3 neurons (Aniksztejn et al., 1995); blockade of mGluR blocks LTP in fields CA3 and CA1 (Bashir et al., 1993).

We also targeted dantrolene-sensitive storage sites and sites regulated by IP_3 to examine their participation in calcium-dependent potentiation induced by forskolin. Release of calcium from dantrolene-sensitive intracellular stores in the SER has been found to elevate calcium to levels required for a number of physiological processes in hippocampal neurons (Belousov et al. 1995; Krnjevic and Xu, 1996), including LTP and LTD (O'Mara et al., 1995). We found that

pretreatment with 20 μM dantrolene did not affect forskolin potentiation.

The disparity in the actions of dantrolene versus TMB-8 was not unusual. In cultured cerebellar granule cells, TMB-8, but not dantrolene, was found to exert a protective effect against the calcium-dependent component of glutamate-induced neurotoxicity (Malcolm et al., 1996). The precise mechanism of TMB-8 action is still in question. Published reports support diverse actions of TMB-8 (Janis et al., 1987). Low concentrations (1 nM to 1 μM) allosterically modulate activation of muscarinic cholinergic receptors in a variety of cell types; high concentrations (>300 μM) can be toxic to neurons (Gnagey and Ellis, 1996; Bencherif et al., 1995). We found that TMB-8 at concentrations less than 20 μM had no effect on forskolin potentiation. Therefore, we conclude that inhibition of muscarinic receptor function does not affect potentiation induced by forskolin. At ≥100 μM, we saw a striking reduction in potentiation during forskolin application, and a complete blockade upon washout; at this concentration there was a notable decrease in transmission with prolonged exposure to the drug. In summary, we attribute the effect of 50 μM TMB-8, which is below toxic levels, to interference with IP_3 receptors.

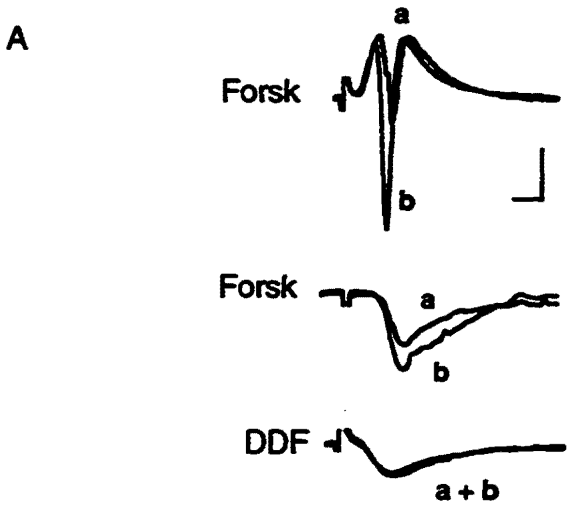
Forskolin-Induced Potentiation Requires Calcium from Extracellular and Intracellular Sources

Complete blockade of the potentiation produced by forskolin at the medial perforant path-granule cell synapse was unacheivable by any of the pharmacological agents utilized in this study. However, it should be noted that there is no other published work in any hippocampal field that demonstrates complete blockade of the initial response to forskolin.

Our data indicate that extracellular calcium entering through NMDA receptor channels and L-type calcium channels, as well as intracellular calcium released from IP_3 receptor-sensitive stores, all contribute to forskolin-induced potentiation. Evidence exists for cooperativity between membrane-bound receptors and SER-bound receptors in LTD (Kasono and Hirano, 1995; Reyes and Stanton, 1996) and in the function of Ca^{2+}-activated K^+ channels (Shirasaki et al., 1994; Imanishi et al., 1996) in the hippocampus. Activation of PKA enhances IP_3 receptor-mediated calcium flux, but this event is highly dependent on calcium concentration in the local environment (Henzi and MacDermott, 1992).

The experimentally imposed global activation of adenylyl cyclase by forskolin focuses on the role of cAMP in long-lasting potentiation. We have utilized this approach to examine the involvement of various mediators of calcium fluxes and their contribution to induction and maintenance of forms of plasticity expressed following elevation of intracellular cAMP.

Figure 1. Forskolin induces a potentiation in dentate gyrus that is mediated by cAMP. **A**, *Traces* are representative examples of evoked population spike (upper traces) and EPSP middle traces) recorded 5 min before forskolin application (**a**) and 60 min after washout of forskolin (**b**). **B**, Time course of potentiation induced by bath application of 50 μM forskolin (*closed squares*, $n = 5$). Forskolin was applied in the 20 min period indicated by the solid bar. Also represented in the same graph is the lack of effect of 50μM 1,9-dideoxyforskolin (DDF) on EPSP slope (*open diamonds*, $n = 3$), seen also in lower traces in *A*. Each point is the mean ± SEM. In all figures, the left end of the horizontal bar is positioned at the beginning of the arrival of forskolin to the slice. The letters "**a**" and "**b**" in bold type indicate time points from which raw data have been taken for exemplary purposes. (Calibration: 2.5 mV/2 ms for all figures.)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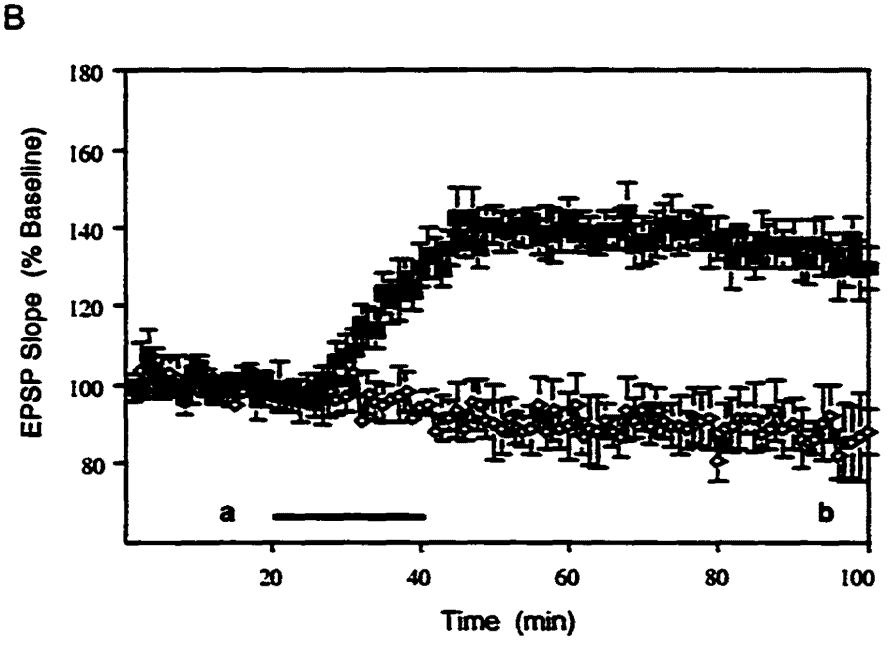
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Time (min)

Fig. 1

Figure 2. Long-lasting potentiation by forskolin is not due to slow washout of DMSO-solubilized agent. Time course of forskolin-induced potentiation by bath application of 50 μM 6β-[β'-(piperidino)-propionyl]- HCl, a water-soluble analog of forskolin (n = 4). Duration of application was 20 min, indicated by the *solid bar*. Representative traces recorded at times **a** and **b** are shown as an inset.

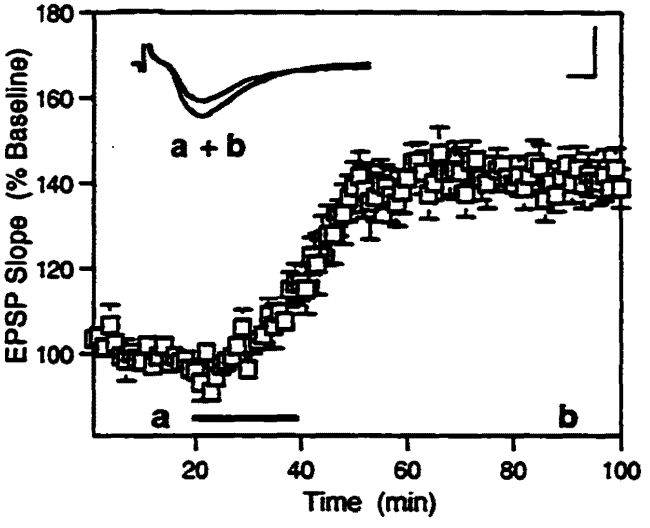
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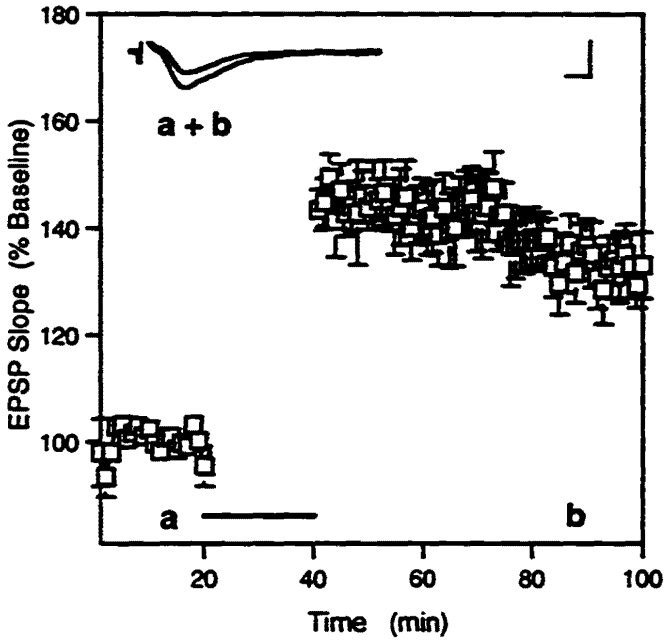
Fig. 2

Figure 3. Stimulation of presynaptic fibers is not required for the expression of forskolin-induced potentiation. Time course of experiments (n = 5) in which perfusion of slices with 50 μM forskolin was carried out in the absence of low frequency (test) stimulation of the medial perforant path inputs to the dentate granule cells. The initial slope of the EPSP reaches a maximum by the end of the application period (as indicated by the *solid bar*), and remains elevated for at least 60 min after washout of forskolin. Each point is the mean ± SEM.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EPSP Slope (% Baseline)
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Time (min)

Fig. 3

Figure 4. Calcium influx from distinct sources modulates forskolin-induced potentiation. ***A***, Nifedipine and D-APV reduce the potentiation induced by forskolin. Forskolin potentiation is demonstrated by the open triangles; these data were used for comparisons in *Fig. 4A*, *4B* and *4C*. Dependence of forskolin-induced potentiation on influx of calcium from extracellular sources was investigated by a 20 min pretreatment with either 20 μM D-APV (A, open squares, n = 7), 10μM nifedipine (N, open circles, n = 8) or 20μM D-APV and 10μM nifedipine together (A + N, open diamonds, n = 8). Exposure to inhibitors was continued during and in the 20 min following perfusion of forskolin (forskolin perfusion is indicated by the *solid bar*). This time course analysis demonstrates that in the presence of D-APV or nifedipine, potentiation was reduced. *Inset traces* are representative examples taken after 20 min of baseline (forskolin) or at the end of pretreatment with A, N or A + N (**a**), and 60 min after washout of forskolin (**b**). Each point is the mean; SEM is indicated every 10 min. ***B***, Interference with release of intracellular calcium via the IP_3 receptor decreases the long-lasting forskolin-induced potentiation. A concentration-dependence study was carried out using 20 μM (open squares, n = 8) and 50 μM (open diamonds, n = 4) TMB-8, an agent that blocks release of calcium from the endoplasmic reticulum mediated by activation of the IP_3 receptor. The *solid bar* indicates the period of forskolin application. *Inset traces* are representative sweeps collected 5 min before addition of, and 60 min after washout of forskolin. Increasing the concentration of TMB-8 reduces the initial rise in the EPSP slope and the long-lasting effect of potentiation. Each point represents the mean; SEM is shown for every tenth point. ***C***, Summary of data from *4A* and *4B*. None of the inhibitors has a significant effect on basal transmission (drug 20 min); all reduce potentiation following washout of forskolin (wash 60 min). Forskolin alone, white bars (50 μM, n = 5); D-APV, gray bars (20 μM, n = 7); nifedipine, black bars (10 μM, n = 8); A + N, double-slashed bars (20 μM + 10 μM respectively, n = 8); TMB-8, light-hatched bars (20 μM, n = 8); TMB-8, dark-hatched bars (50 μM, respectively, n = 4). Each bar is the mean ± SEM. Asterisk indicates significant difference from forskolin alone (P < 0.05).

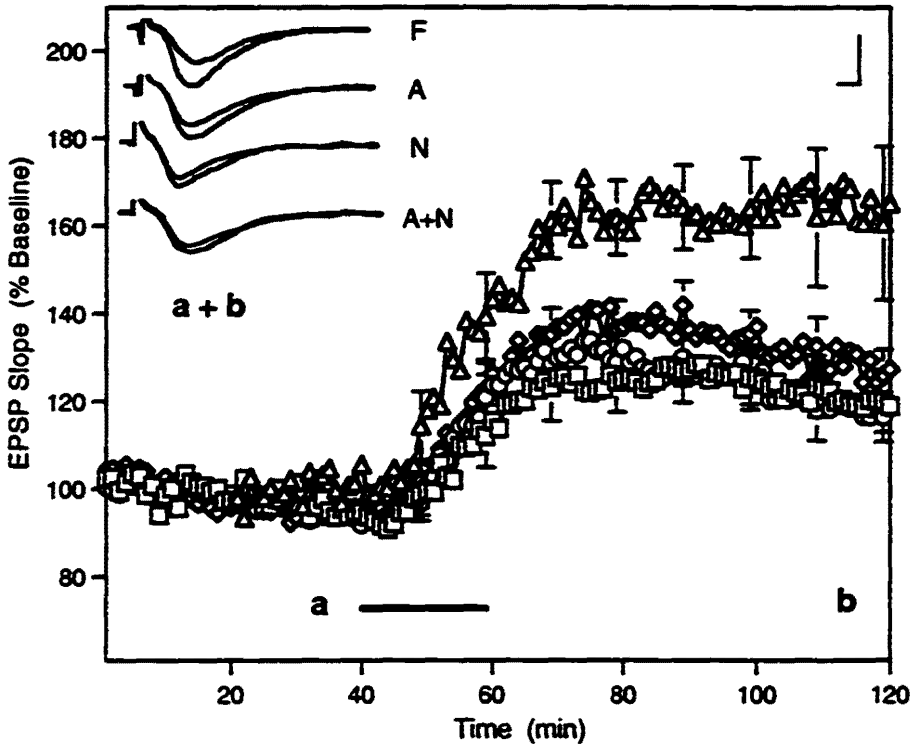
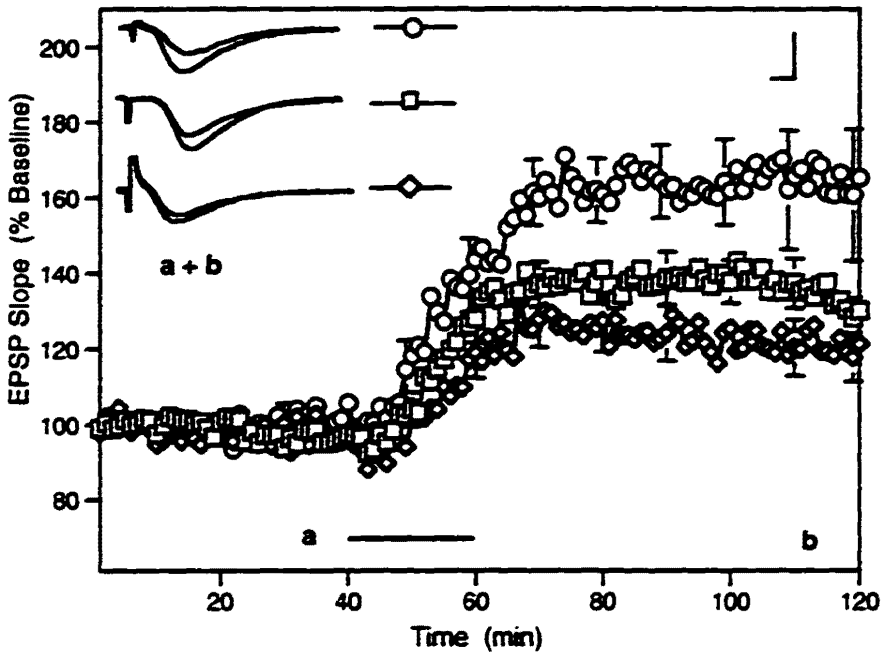

F
A
N
A+N
a + b
EPSP Slope (% Baseline)
200
180
160
140
120
100
80
a
b
20
40
60
80
100
120
Time (min)

Fig. 4A


EPSP Slope (% Baseline)
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a + b
a
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100
120
Time (min)

Fig. 4B

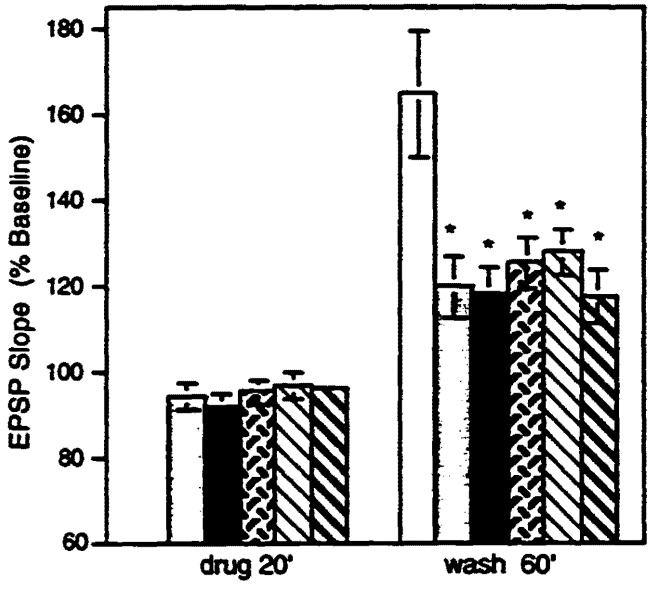

Fig. 4C

Figure 5. Calcium-induced calcium release does not contribute to forskolin-induced potentiation. Dantrolene (20 μM) was bath-applied for 60 min prior to introduction of forskolin (*solid bar*) into the bath (n = 4). This agent had no demonstrable effect on the maintenance of potentiation by forskolin. *Inset traces* show examples of sweeps taken at points **a** and **b**. Each point is the mean ± SEM.

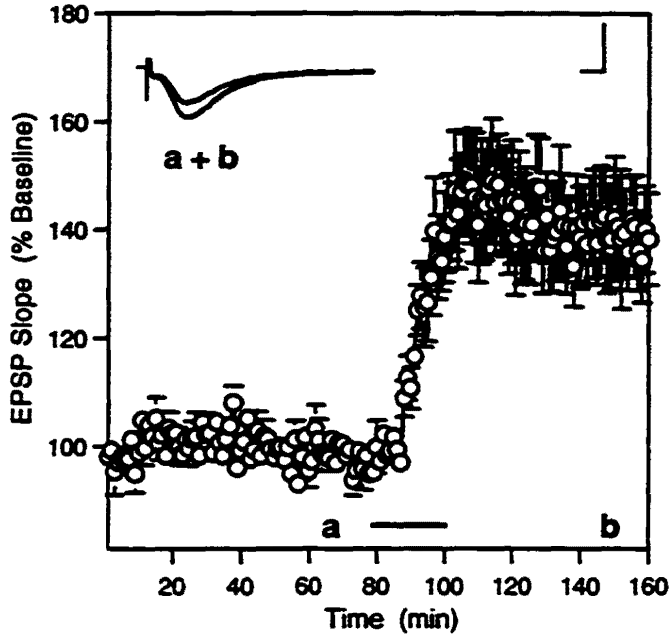
180
160
140
120
100
EPSP Slope (% Baseline)
a + b
a
b
20
40
60
80
100
120
140
160
Time (min)

Fig. 5

A Method for Isolating Slices of Hippocampal Dentate Gyrus for Electrophysiological Analysis

Summary

We have developed a simple technique to isolate slices of dentate gyrus. The preparation is useful for analyzing biochemical changes occurring in the dentate gyrus as a result of either chemical or electrical stimulation. Hippocampi are dissected from rat brain and trimmed to remove entorhinal cortex and CA3. The remaining tissue is composed of dentate gyrus and CA1, from which slices are cut and placed in an interface recording chamber. Slices are allowed to adhere to filter paper, followed by removal of CA1. After a period of equilibration, electrophysiological analyses follow. By applying criteria that we normally use for selecting slices for electrophysiological experiments, we have demonstrated that these dentate minislices are as healthy as intact hippocampal slices. Dentate minislices can support long-term potentiation (LTP) of the population spike and the excitatory post-synaptic potential (EPSP). Combining this technique with the use of an interface chamber, one can generate a large amount of tissue from a specific hippocampal region that has been stimulated electrically or chemically under identical conditions. This experimental design insures a greater signal-to-noise ratio when conducting subsequent biochemical analyses on such tissue.

Keywords: Dentate Gyrus; Hippocampal slices; Long-term potentiation; Electrophysiology; Biochemistry

1. Introduction

The *in vitro* brain slice preparation has become an essential tool for conducting electrophysiological and pharmacological studies. In particular, the hippocampal slice is the primary preparation with which long-term potentiation is studied (Andersen et al., 1977; Nicoll and Malenka, 1995; Stanton and Sarvey, 1984; Sarvey et al., 1989). As the fields of molecular biology, biochemistry, pharmacology and electrophysiology continue to converge, it has become necessary to combine different technical approaches in order to understand mechanisms underlying cellular phenomena. For example, methodological approaches are becoming more intricate as investigators examine synaptic plasticity using electrophysiological, followed by molecular and biochemical analyses of stimulated neuronal tissue.

One of the difficulties inherent in conducting such analyses using the hippocampal slice preparation is the problem of isolating the cell population of interest. For example, a number of studies examining long-term potentiation in the hippocampal area CA1 have been carried out by stimulating and rapidly freezing tissue on dry ice where the challenge is then to cut out CA1 without sending pieces of frozen tissue flying in all directions (English and Sweatt, 1996). In our studies in which we examined changes in the phosphorylation state of key regulatory proteins, we found it

necessary to isolate the dentate gyrus (Voulalas and Sarvey, 1995a).

The anatomical organization of the hippocampus consists of a tri-synaptic pathway (dentate gyrus > CA3 > CA1) (Andersen, 1975). Thus, one could hypothesize that isolating any stimulus to a single pathway would be difficult. In addition, one must consider the alternative situation, whereby unstimulated regions of the slice could weaken a biological signal measured in a single pathway, as a result of stimulating that particular pathway. For this reason, we developed a method of slice preparation such that the dentate gyrus is completely dissected free from the CA3 and CA1 areas. The procedure is relatively simple, involving two additional steps that, although somewhat time-consuming, result in the generation of minislices that by our criteria are as healthy as the intact hippocampal slice. Besides possessing normal characteristics of transmission, these minislices are capable of exhibiting long-term potentiation. Using this preparation, one can generate enough samples to conduct a time course analysis of biochemical changes following stimulation.

2. Materials and methods

Male Sprague-Dawley rats (Taconic, Germantown, N.Y.) weighing 80-180g were anesthetized with ketamine hydrochloride (100mg/kg i.p.) and decapitated. The brain was rapidly removed and placed in cold artificial cerebrospinal fluid (ACSF) for dissection of the hippocampi. The ACSF contained, in mM: $NaHCO_3$ 26, NaCl 124, KCl 1.75, KH_2PO_4 1.25, $MgSO_4$ 1.3, $CaCl_2$ 2.4, dextrose 10, and pH was adjusted to 7.4 by bubbling with 95% CO_2/5%O_2. Once removed, the hippocampus was placed on the block of a McIlwain tissue chopper, where the rostral and caudal thirds of the hippocampus were trimmed off with a razor blade (Fig. 1). After field CA3 and the entorhinal cortex were removed, the middle third of the hippocampus consisted of dentate gyrus and field CA1, from which 400μm thick slices were chopped. The dentate/CA1 minislices were placed on ACSF-saturated filter paper on the stage of a modified Oslo interface chamber. Slices were perfused with ACSF at a rate of 3ml/min, 32-34°C, and allowed to "settle" onto the paper for one hour. Then, the CA1 area was dissected with a micro scalpel, approximately 100μm from the hippocampal fissure. The dentate minislices were then allowed to equilibrate for at least two hours, at which time electrophysiological analyses were begun.

Responses were generated by stimulating the medial perforant path fibers (middle third of the molecular layer) with a monopolar electrode (75μm diameter Teflon-insulated

stainless steel wire, exposed only at the tip). Extracellular recordings were made using glass microelectrodes filled with 2M NaCl (2-10MΩ). Recording electrodes were positioned a minimum of 500μm from the stimulating electrodes, and lowered to a final depth of 80 to 120μm into the slice. Population spikes were recorded in the granule cell body layer and excitatory post-synaptic potentials (EPSP) were recorded in the mid-molecular layer, within the pathway innervated by medial perforant path fibers (Dahl and Sarvey, 1989). Confirmation of medial perforant path activation was achieved by paired-pulse stimulation using current and stimulus durations that yielded an EPSP that was sub-threshold for eliciting a reflected population spike. With an inter-pulse interval of 80ms, the medial perforant path EPSP is characterized by a lack of facilitation, and often a depression of the second response (McNaughton, 1980; Colino and Malenka, 1993; Bramham and Sarvey, 1997). An input-output curve was generated for the population spike using stimulus durations of 20μs to 200μs; current was adjusted such that a pulse of 20μs duration was sub-threshold for generation of a spike. Population spikes were evoked and recorded throughout the experiment using a duration that yielded a spike amplitude that corresponded to 30% of the maximal response. Evoked EPSPs were recorded using a duration that corresponded to 20 to 30% of the maximal EPSP slope. Long-term potentiation was induced by sending a 100Hz, 2s train of current of a duration that

yielded a population spike that was 50% of the maximal response.

Responses were amplified, filtered (DC to 3kHz) digitized at 20kHz (DAS-20 interface, Keithley Metrabyte, Taunton, MA) and stored for analysis using the Labman data acquisition and analysis program (generous gift of Dr. T. Teyler, NeuroScientific Laboratories, Rootstown, OH). Measures of synaptic efficacy were made using the initial slope of the EPSP and the amplitude of the population spike. Values were normalized to percentages of the mean baseline value. In order for evoked responses to meet the criterion of "potentiated," the magnitude of the response measured 60 minutes after the high frequency stimulus was delivered must exceed the mean baseline value by more than two standard deviations (Stanton and Sarvey, 1984).

3. Results and discussion

As shown in figure 2A, the strict criteria we have routinely used to select intact hippocampal slices for electrophysiological analysis were met in these minislices. The following characteristics have indicated a "healthy" intact slice to be chosen for study. First, the population evoked potential at the cell body layer should yield a single spike ranging from 4 to 8mV in amplitude; The representative slice in Fig. 2A had a maximal evoked spike of 7.2mV. Next, we regularly verify intact inhibitory inputs to the dentate granule cells by paired-pulse stimulation. The half-maximal population spike should demonstrate greater than 90% depression when pulses are paired with a 20ms inter-pulse interval, and should remain depressed when paired with a 30ms inter-pulse interval. In this slice, the half-maximal spike was depressed by 94% at 20ms IPI and 64% at 30ms IPI. Third, the amplitude of the EPSP recorded in the mid-molecular layer should be 3 to 6mV before a reflected spike is observed. The EPSP recorded in the dendrites had a 5.4mV amplitude, sub-threshold for a reflected spike. Finally, isolation of medial perforant path is typically confirmed by either no change in, or depression of the initial slope and amplitude of the EPSP when pulses are paired with an 80ms inter-pulse interval. This is demonstrated in Fig. 2A, where the slope was depressed by 35%. Therefore, our technique, involving a

modification of the standard hippocampal slice preparation, resulted in the generation of minislices that met our electrophysiological standards.

As shown in figure 2B, high frequency stimulation delivered to the medial perforant path fibers resulted in a long-lasting increase in the population spike amplitude. It also led to a substantial increase in the initial slope of the EPSP, as recorded in the mid-molecular region in minislices. The magnitude of LTP one hour after tetanus met the requirement of being two standard deviations greater than baseline values. Paired pulse stimulation to assess inhibition of the population spike at the end of the experiment yielded 86% depression of the second spike at a 20ms inter-pulse interval, and 49% depression at a 30ms inter-pulse interval. Such robust responses are comparable to what is normally achieved with an intact hippocampal slice (Burgard et al., 1993).

The minislice preparation has allowed us to examine events specifically taking place in the dentate gyrus, increasing the signal-to-noise ratio by eliminating biochemical signals in the CA1 and CA3 regions occurring as a result of stimulation. With chemical stimulation of intact slices, activation of neurons throughout the slice is obviously unavoidable. Secondary effects of interactions of the dentate granule cells with other areas of the hippocampus also occur when slices are electrically stimulated. For example, current spread across the hippocampal fissure into

the stratum lacunosum-moleculare of CA1 may result from stimulation of the perforant path and could very well activate the CA1 pyramidal cells. Due to the trisynaptic circuit, antidromic and feedback activation of CA3 pyramidal cells can occur via the Schaffer collaterals and commissural fibers of CA1 (Schwerdtfeger and Sarvey, 1983; Scharfman, 1994). Finally, feedback from CA3 to the dentate granule cells can also activate the latter.

The hippocampal slice preparation has provided a technical approach that is intermediate between primary neuronal cultures and the in vivo rat. The limitation of primary cultures as a model system is the lack of real neuronal networks that exist in the slice or the intact brain. The limitation of the in vivo, whole animal preparation is the inability to carry out pharmacological analyses as easily as in the in vitro slice preparation. Although the minislice still consists of more than the pre- and postsynaptic elements of the dentate gyrus (e.g., the hilar interneurons, glia and interneurons within the molecular layer of the dentate are still intact), we have eliminated two other major neuronal populations, the pyramidal cells of fields CA3 and CA1.

An important step in the procedure was the period during which the minislices were allowed to adhere to the paper. This stabilized the tissue and facilitated the final dissection of CA1. The two hour period of equilibration following the removal of CA1 was also critical. Initiation

of recordings without first allowing the manipulated slices to recover resulted in responses that were quite unstable over time.

We have used this technique to generate up to 100 minislices per experiment for molecular and biochemical analysis, with all 100 slices being incubated in an interface chamber designed to hold a large number of tissue slices.

In conclusion, we have confirmed the ability to further dissect a hippocampal slice to isolate a specific region that is electrophysiologically robust. This tissue can then be used for a variety of biochemical analyses including Western blotting and assays for enzymatic activity.

1. Flow diagram for preparation of minislices. The upper portion of the figure shows the left hippocampus following dissection from the rest of the brain. See text for detailed description.

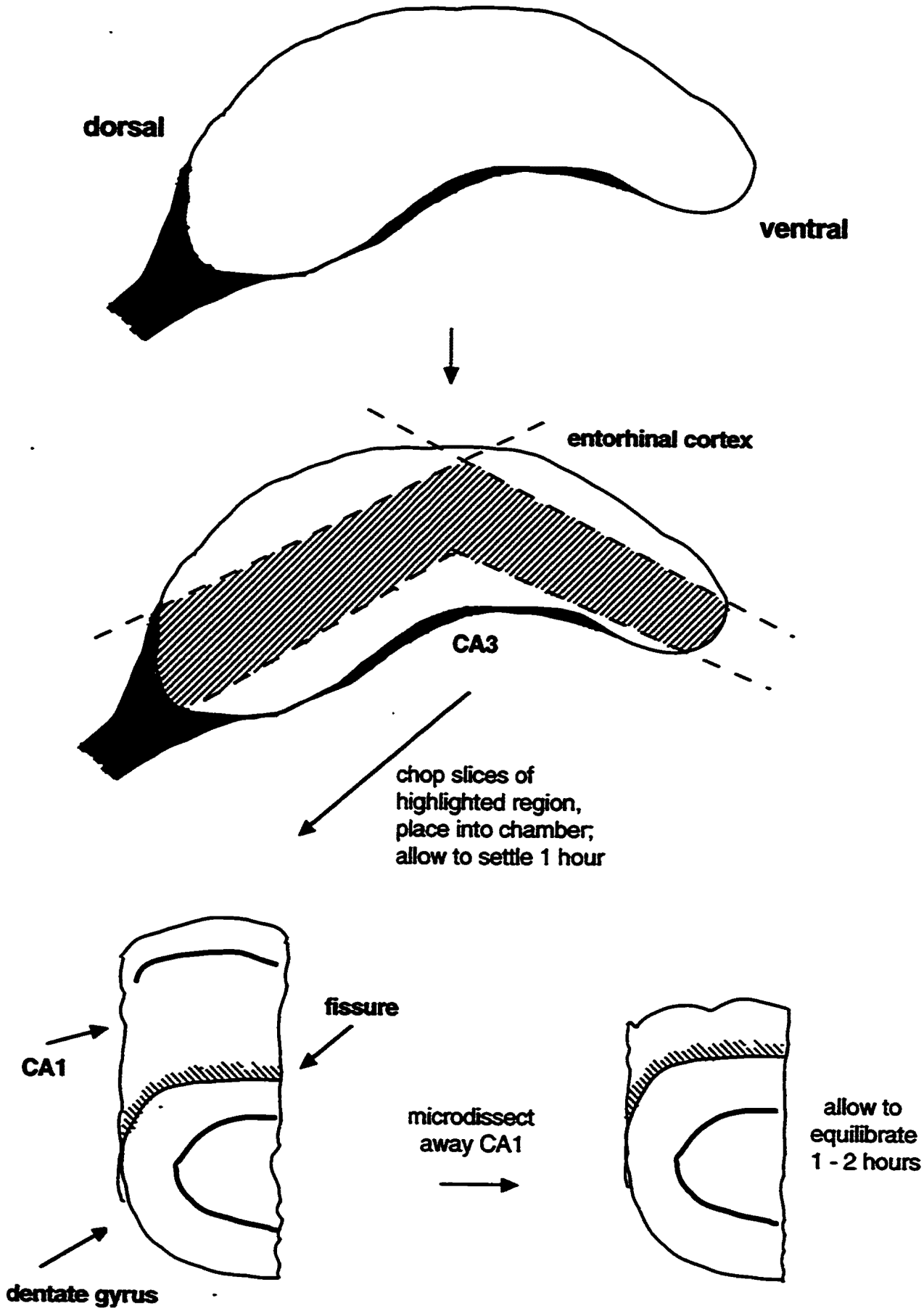
dorsal
ventral
entorhinal cortex
CA3
chop slices of
highlighted region,
place into chamber;
allow to settle 1 hour
fissure
CA1
dentate gyrus
microdissect
away CA1
allow to
equilibrate
1 - 2 hours

Fig. 1

2. Minislices demonstrate robust evoked responses and paired-pulse depression. Depression of the population spike is observed when pulses are paired with a (A) 20ms and (B) 30ms inter-pulse interval. C: Isolation of a medial perforant path EPSP is demonstrated by depression of the second response when pulses are paired with an 80ms inter-pulse interval. Solid lines indicate response to first pulse; dashed lines indicate the response to the second pulse.

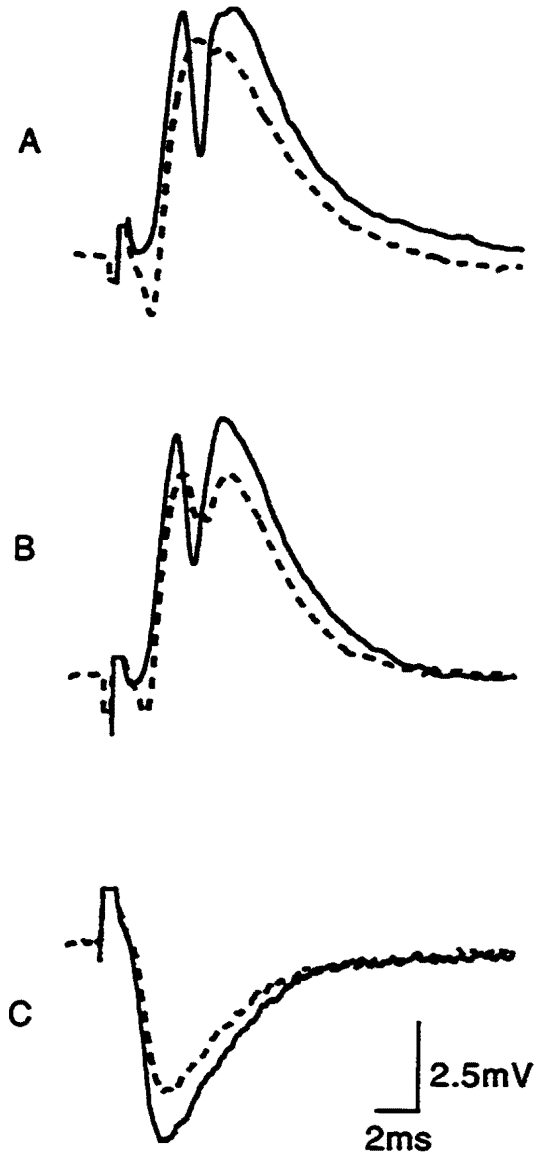

Fig. 2

3. Long-term potentiation is observed in minislices. A: Time course of LTP as measured in the population spike amplitude. B: Time course of potentiation of the initial slope of the EPSP. Arrows indicate the point at which high frequency stimulation was delivered. Inset traces show a response collected before the HFS (solid line) and 60 minutes after potentiation was induced (dashed line). Calibration bars correspond to 2.5mV/2ms.

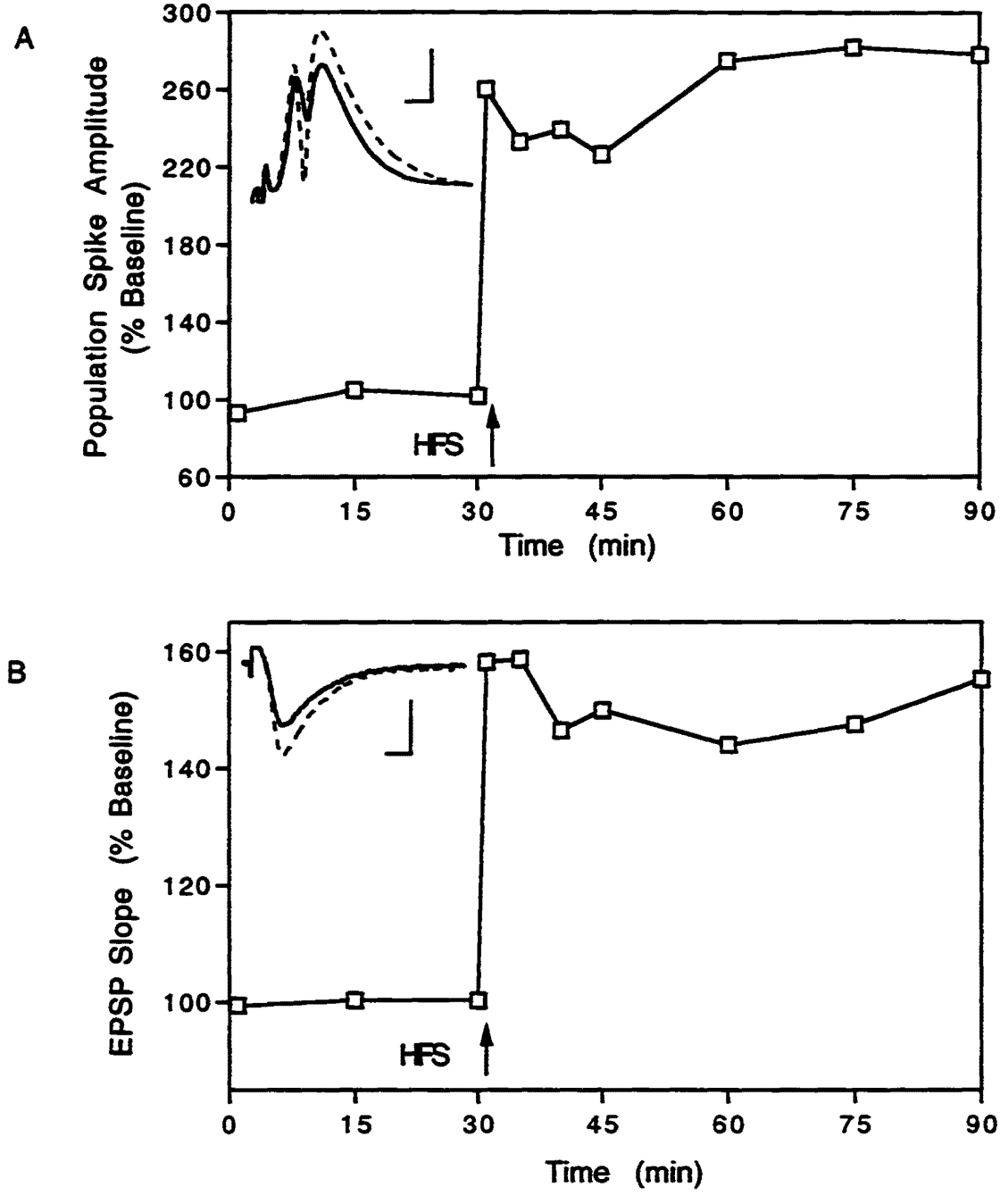
A
Population Spike Amplitude
(% Baseline)
300
260
220
180
140
100
60
HFS
0
15
30
45
60
75
90
Time (min)
B
EPSP Slope (% Baseline)
160
140
120
100
HFS
0
15
30
45
60
75
90
Time (min)

Fig. 3

Activation of the cAMP Response Element-Binding Protein (CREB) and Mitogen-Activated Protein Kinases (MAPKs) by cAMP-Dependent Potentiation in the Hippocampus

SUMMARY

Activation of CREB and the MAPKs has been implicated in plasticity, but their precise roles are still under examination. We investigated the time course and pharmacological sensitivity of activation of CREB and MAPKs in a cAMP-dependent form of synaptic plasticity in the hippocampal dentate gyrus. Using phospho-specific antibodies, we demonstrated that forskolin induced a long-lasting potentiation of medial perforant path-granule cell EPSPs and also increased phosphorylation of p42 (ERK-2) and p44 (ERK-1) MAPK and CREB in the dentate gyrus. Phosphorylation of CREB and ERK-1 and 2 peaked within 15 to 20 min of forskolin application; dideoxyforskolin neither potentiated responses nor increased phosphorylation. The tyrosine kinase inhibitors lavendustin A and methyl 2,5-dihydroxycinnamate (MDC) and the MAPK kinase (MEK) inhibitor PD098059 reduced potentiation and blocked CREB phosphorylation. PD098059, but not MDC, blocked ERK phosphorylation. NMDA receptor blockade reduced forskolin potentiation but did not prevent forskolin-induced ERK phosphorylation. Blocking NMDA receptors decreased basal CREB phosphorylation, and also blocked the rise following forskolin perfusion. Inhibition of IP_3 receptor function with TMB-8 strongly depressed forskolin-induced potentiation and prevented CREB phosphorylation, but did not block increases in ERK phosphorylation. We suggest that the diverse effects of these inhibitors on CREB and ERK phosphorylation reflect a

role for calcium from several sources, and a requirement for MAPK activation for potentiation as well as CREB activation during potentiation.

INTRODUCTION

Although a detailed description of the cellular mechanisms underlying information storage and retrieval in the hippocampus is lacking, many important molecular modulators in the process have been identified. The second messengers calcium and cAMP are critical for eliciting changes in synaptic strength (Sarvey et al., 1989; Frey et al., 1993; Blitzer et al., 1995; Collingridge and Bliss, 1995). Membrane-to-nucleus signaling involving the cAMP response element-binding protein (CREB) and the mitogen-activated protein kinases (MAPKs) is a focal point of this research, as these messengers are able to transduce increases in calcium and cAMP to increases in protein synthesis (Ghosh et al., 1994). Protein synthesis is believed to provide the physical basis for stable formation or modification of synapses (Stanton and Sarvey, 1984; Deadwyler et al., 1986; Geinisman et al., 1996).

Abundant evidence supports a role for CREB in hippocampal synaptic plasticity (Bourtchuladze et al., 1994; Deisseroth et al., 1996). Activation of CREB by phosphorylation on Ser-133 is triggered by stimulation of membrane receptors that elevate intracellular calcium, cAMP or both (Gonzalez and Montminy, 1989; Dash et al., 1991; Sheng et al., 1991).

The MAPKs are serine/threonine kinases that are primary transducers of growth factor receptor signals. In the brain,

examples include activation of Trk receptors by the neurotrophins NGF, BDNF, and NT-3. These signals are initiated via Ras and the Rafs, and lead to the activation of MAPK kinase (MEK). MEK is a dual-specific kinase which activates MAPKs by phosphorylating the proteins on threonine and tyrosine residues (Campbell et al., 1995). MAPKs phosphorylate and activate $pp90^{RSK}$ (Sturgill et al, 1988), and this function of the MAPKs has been linked to protein synthesis, most recently by virtue of $pp90^{RSKII}$ being identified as a CREB kinase (Ginty et al, 1994).

The phenomenon of long-term potentiation (LTP) is a useful model for learning and memory in the hippocampus (Bliss and Lømo, 1973). Expression of LTP in the dentate gyrus is known to involve increases in levels of the second messenger cAMP, and a form of synaptic plasticity induced by elevating cAMP levels in the hippocampus has been described (Dunwiddie et al., 1992; Huang et al., 1994; Voulalas and Sarvey, 1997b). Bath application of forskolin induces long-lasting potentiation in the hippocampus that is protein synthesis-dependent (Huang et al., 1994). We have used this model to obtain details about the pathways activated when synaptic responses are potentiated in a cAMP-dependent manner. The mechanism whereby elevation of cAMP alone can potentiate responses is not well understood. Here we describe the time course of activation of CREB and the MAPKs ERK-1 and ERK-2 by forskolin and characterize the modulation by kinases and calcium of these phosphorylation events.

MATERIALS AND METHODS

Materials - Forskolin and 1,9-dideoxyforskolin were purchased from Calbiochem. Lavendustin A, methyl 2,5-dihydroxycinnamate (MDC) and PD098059 were obtained from LC Labs (Woburn, MA). D-APV was purchased from Tocris-Cookson (Ballwin, MO). Aprotinin, leupeptin and pepstatin were obtained from Boehringer Mannheim. Sodium orthovanadate, disodium molybdate, glycerol-2-phosphate, benzamidine, sodium fluoride and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. BSA (Fraction V) was purchased from United States Biochemical Corp. Buffer reagents for electrophysiological experiments were obtained from Fluka. Anti-Active MAPK antibody was purchased from Promega and anti-ERK-1 antibody was purchased from Santa Cruz. Sources of anti-phosphoCREB were New England Biolabs and M.E. Greenberg, who also generously provided an anti-phosphoMAPK antibody.

Preparation of Hippocampal Slices and Electrophysiological Recordings - Male Sprague Dawley rats (Taconic, Germantown, N.Y.) weighing 100-200 g were anesthetized with ketamine hydrochloride (100 mg/kg i.p.) and decapitated. Transverse slices (400 μm) of hippocampus were prepared using a McIlwain tissue chopper and placed in a modified Oslo interface recording chamber at 32-34° C. Slices were perfused at 2.5 - 3 ml/min with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 1.75, KH_2PO_4 1.25, $MgSO_4$ 1.3, $NaHCO_3$ 26, $CaCl_2$ 2.4, and dextrose 10; bubbling with 95% CO_2/5% O_2

resulted in a pH of 7.2 - 7.4. Slices were equilibrated for at least two hours before recordings were initiated. Medial perforant path fibers (middle third of the molecular layer) were stimulated with a monopolar electrode (75 μm diameter Teflon-insulated stainless steel wire, exposed at the tip). Standard glass micropipettes filled with 2 M NaCl, 3-8 MΩ resistance were used to obtain extracellular recordings. Stimulating electrodes were kept a minimum of 500 μm from the recording electrodes; recording electrodes were lowered 80 to 120 μm into the slice (Dahl and Sarvey, 1989; Bramham and Sarvey, 1997). Evoked EPSPs (50% of maximum amplitude; 1-2 mV and subthreshold for a reflected spike) were recorded in the mid-molecular layer of the dentate gyrus. Test stimuli were delivered every 30 s.

For Western blot analyses, dentate minislices were prepared (Voulalas and Sarvey, 1997). A single group of 2 - 3 animals for each time course was used to generate minislices. All were incubated simultaneously in the same chamber to minimize variation between preparations. Stimulated tissue was collected at specific times and rapidly frozen in liquid nitrogen, then stored at -80°C to await processing. Application of drugs was started only after at least 20 min of stable baseline recording.

Electrophysiological recordings were amplified, low-pass filtered (3 kHz) digitized at 20 kHz (DAS-20 interface, Keithley Metrabyte, Taunton, MA) and stored for analysis using the Labman data acquisition and analysis program

(generous gift of Dr. T. Teyler, NeuroScientific Laboratories, Rootstown, OH). Synaptic efficacy was measured using the initial slope of the EPSP. Initial slope after 60 min washout of forskolin was normalized to percentage of the mean baseline value.

Statistical analysis of drug and forskolin effects was carried out in Statview (Abacus Concepts, Berkeley, CA) using the paired t-test, Student's t-test or ANOVA plus a post hoc Bonferroni/Dunn test for multiple comparisons. A probability of 0.05 was selected as the level of statistical significance. All data are expressed as the mean ± SEM.

Drug application - Drug application was achieved by switching the chamber infusion to ACSF containing the drug. Forskolin was applied for 20 min; application of inhibitory compounds preceded forskolin by 20 min, and were continued during and for 20 min after the treatment with forskolin; PD098059 was applied for 30 min prior to forskolin application. Experiments with and without antagonists were grouped and compared taking into account the batch of forskolin used and the time-frame of usage. All agents were made up as 1000-fold stock solutions stored at -20° C and diluted just prior to use. Forskolin, 1,9-dideoxyforskolin, MDC, PD098059 and lavendustin A were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO never exceeded 0.2%, which had no effect on responses (Voulalas and Sarvey, 1997b). D-APV and TMB-8 were dissolved in water.

Preparation of dentate gyrus extracts and Western blot analyses. Dentate gyrus tissue (20 - 50 mg) in 250-500µl of 10mM Tris, pH 7.4 was homogenized with 12 strokes in a tight fitting glass/Teflon homogenizer. Aprotinin (2µg/ml), leupeptin (0.5µg/ml), pepstatin (7µg/ml), sodium orthovanadate (0.2mM), disodium molybdate (5mM), glycerol-2-phosphate (25mM), benzamidine (10mM), sodium fluoride (50mM) and PMSF (0.5mM) were added to minimize protease and phosphatase activity. The BCA Protein Assay Kit (Pierce) was used to quantify protein and to insure equivalent loading of polyacrylamide gels. Equal loading was confirmed by Ponceau S staining of Western blots.

Whole cell extracts from slices of dentate gyrus were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Antibodies were titrated with serial dilutions of extract; immunoreactivity increased proportionally from 1 to 20 µg protein (data not shown). Blots were blocked with 10% BSA/TBS-Tween, and antibody incubations were done in 1% BSA/TBS-Tween. An HRP-conjugated secondary antibody followed by enhanced chemiluminescence (ECL, Amersham) was used to detect immunoreactive proteins. Quantitation of Western blots was carried out using Quantity One (PDI). Statistical analysis of drug and forskolin effects on CREB and MAPK phosphorylation was carried out in Statview using the paired t-test or ANOVA plus a post hoc Bonferroni/Dunn test for multiple comparisons. A probability

of 0.05 was selected as the level of statistical significance. All data are expressed as the mean ± SEM.

RESULTS

Forskolin induces a long-lasting potentiation in the dentate gyrus and activates CREB, ERK-1 and ERK-2 - Chemically inducing potentiation in hippocampal slices by elevating cAMP has been used to study the effects of protein kinase A (PKA) activation in hippocampal slices (Dunwiddie et al., 1992; Huang et al., 1994; Voulalas and Sarvey, 1997b). As demonstrated in Fig. 1, bath application of 50 μM forskolin gave rise to potentiation of the initial slope of the medial perforant path-granule cell EPSP in the dentate gyrus that outlasted the application of forskolin (133 ± 6%, n = 5, P < 0.05, paired t-test). Perfusion of slices with the inactive analog of forskolin, 50 μM dideoxyforskolin (Fig. 1) did not potentiate EPSPs (85 ± 13%, n = 3, P > 0.05, paired t-test). Dideoxyforskolin yielded a slight depression of the responses, characteristic of the non-specific effect of forskolin and its analogs on a calcium-mediated potassium conductances (Hoshi et al., 1988; Harris-Warrick, 1989).

Perfusion of dentate gyrus minislices with 50 μM forskolin stimulated an increase in phosphorylation of CREB over basal levels (167 ± 15% of baseline 10 to 20 min after start of forskolin perfusion, n = 9, P < 0.05, paired t-test). Samples were removed at various times before, during and after forskolin application. Fig. 2A shows the results of a typical experiment characterizing the time-course of CREB phosphorylation using a Western blot probed with an anti-

phosphoCREB antibody. A basal level of CREB phosphorylation was always noted. In response to forskolin, CREB phosphorylation increased within 10 min, peaked between 15 and 20 min of forskolin application, and began to decline 15 to 30 min after washout. In contrast to the increases in CREB phosphorylation seen with forskolin, 20 min perfusion of control slices with 50 μM dideoxyforskolin did not increase CREB phosphorylation, as indicated in Fig. 2B (113 ± 11%, n = 3, $P > 0.05$, paired t-test).

The effect of forskolin treatment on ERK phosphorylation is shown in Fig. 3A. Minislices were stimulated for 20 min with 50 μM forskolin, as in Fig. 2A. Using an anti-phosphoMAPK antibody, we detected an elevation in ERK phosphorylation that occurred within 10 min, was maximal during 15 to 20 min of forskolin perfusion, and decreased after 60 min washout of forskolin (ERK-1, 204 ± 21% of baseline 10 to 20 min after start of forskolin perfusion, n = 12; ERK-2, 175 ± 16%, n = 11, both $P < 0.05$, paired t-test). Control slices treated with dideoxyforskolin did not increase ERK phosphorylation over basal levels (Fig. 3B; ERK-1, 120 ± 6%, n = 3; ERK-2, 105 ± 3%, n = 3, both $P < 0.05$, paired t-test).

A tyrosine kinase inhibitor and a MEK inhibitor reduce forskolin-induced potentiation - As seen in Fig 4, forskolin treatment potentiated responses to 186 ± 17% of baseline (n = 4, $P < 0.05$, paired t-test). LTP in the dentate gyrus in vivo is blocked by the tyrosine kinase inhibitors herbimycin and lavendustin A (Abe and Saito, 1993). We have found that 5 μM

lavendustin A blocks LTP and norepinephrine-induced long lasting potentiation in the dentate gyrus in vitro (Voulalas and Sarvey, 1993, 1994). Here we show that 5 μM lavendustin A had no significant inhibitory effect on potentiation induced by forskolin (153 ± 10%, $n = 4$, $P > 0.05$, ANOVA). Lavendustin A inhibits tyrosine kinases by competing with ATP. Therefore, we tested another tyrosine kinase inhibitor with a different mechanism of action. MDC competes with both the substrate and ATP (Imoto et al., 1987; Umezawa et al., 1990). MDC reduced forskolin potentiation to 117 ± 4% ($n = 5$, $P < 0.05$, ANOVA). Forskolin, in the presence of the specific MEK inhibitor PD098059 (Dudley et al., 1995) potentiated responses to only 120 ± 15% ($n = 4$; $P < 0.05$, ANOVA). The observation that the specific MEK inhibitor reduced forskolin potentiation suggests that the MAPKs are necessary for long-lasting potentiation induced by forskolin. The reduction in potentiation seen with MDC implies that tyrosine kinases that are insensitive to lavendustin A are involved in forskolin potentiation.

Effect of two tyrosine kinase inhibitors and a MEK inhibitor on ERK phosphorylation - We investigated the ability of the three kinase inhibitors above to interfere with the activation of ERKs by forskolin. The Western blot analyses conducted in these experiments utilized tissue collected before perfusion with inhibitors, after pretreatment with inhibitor, and at the end of the application of forskolin with inhibitor. Fig. 5 shows that lavendustin A did not inhibit forskolin-induced increases in ERK activation (ERK-1, 159 ± 18%, $n = 3$; ERK-2,

133 ± 8%, n = 3, both $P > 0.05$, ANOVA). The second tyrosine kinase inhibitor used for electrophysiological studies, MDC, prevented increases in ERK phosphorylation, compared to slices treated with forskolin alone (ERK-1, 101 ± 21% , n = 3; ERK-2, 109 ± 5%, n = 3, $P < 0.05$, ANOVA). The specific MEK inhibitor PD098059 decreased basal ERK phosphorylation, and when present with forskolin, produced a significant depression of phosphorylation compared to treatment with forskolin alone (ERK-1, 68 ± 18%, n = 3; ERK-2, 80 ± 6%, n = 3, $P < 0.05$, ANOVA). These data indicate that elevated levels of cAMP contribute to an increase in MAPK activation in the dentate gyrus. Each of the blots was reprobed with an anti-ERK antibody that recognizes total ERK protein, to demonstrate near-equivalent amounts of total ERK loaded in each lane.

Effect of a tyrosine kinase inhibitor and a MEK inhibitor on CREB phosphorylation - In addition to the ability of PKA and calcium/calmodulin kinase II (CaMKII) to phosphorylate CREB, recent evidence suggests that MAPK phosphorylates a CREB kinase that is homologous to $pp90^{RSKII}$, which itself can phosphorylate CREB (Ginty et al., 1994). We asked whether the ability to activate CREB by forskolin was dependent on activation of MAPK. The experimental protocol used in Fig. 6 was identical to that in Fig. 5. In comparison to treatment with forskolin alone, both lavendustin A and MDC prevented forskolin from stimulating CREB phosphorylation (lav A, 77 ± 8%, n = 8; MDC, 106 ± 8%, n = 3, $P < 0.05$, ANOVA). The MEK-specific antagonist PD098059 also reduced forskolin-stimulated

CREB phosphorylation (PD098059, 117 ± 33%, $n = 3$, $P < 0.05$, ANOVA). In view of the fact that lavendustin A blocks CREB phosphorylation without yielding a significant reduction in potentiation, we propose that CREB phosphorylation is not necessary for the first hour of long-lasting potentiation by forskolin. The reduction in CREB phosphorylation by PD098059 strongly implies that MAPKs are necessary, but not sufficient for CREB phosphorylation.

Effect of NMDA receptor blockade on forskolin-stimulated CREB and ERK phosphorylation - While NMDA receptors are required for induction of LTP in the dentate gyrus (Wigström et al., 1986; Errington et al., 1987; Abraham and Mason, 1988; Burgard et al., 1989; Bramham and Sarvey, 1997), we have found that forskolin potentiation in the dentate gyrus is reduced, but not blocked by NMDA receptor antagonists (Voulalas and Sarvey, 1997b). We asked whether this reduction in potentiation was correlated with reduction or blockade of CREB and ERK phosphorylation. To address this issue, we again utilized the same experimental protocol as in Figs. 5 and 6. Minislices of dentate gyrus were removed before application of 20 μM D-APV, 20 min after APV perfusion had been initiated and 20 min after forskolin and APV were applied together. In Fig. 7A, forskolin-stimulated CREB phosphorylation was reduced in the presence of APV, compared to slices stimulated only with forskolin, followed by the inability of forskolin to elicit an increase in phosphorylation (forskolin + APV, 20 min, 68 ± 15%, $n = 3$, $P < 0.05$, ANOVA). Phosphorylation of ERK-1 and 2

induced by forskolin in the presence of APV was not different from phosphorylation induced by forskolin alone, as seen in Fig. 7B (ERK-1, 236 ± 60%, n = 3; ERK-2, 210 ± 73%, n = 3, P > 0.05, ANOVA). Thus, calcium influx through NMDA receptors comprises a substantial contribution to the maintenance of CREB's basal state of activity which appears to affect its ability to respond to subsequent stimulation, while the ERKs are unaffected by NMDA receptor activity in either their basal or stimulated condition.

Effect of IP3 receptor blockade on forskolin-induced potentiation - Assuming that calcium makes an important contribution to potentiation mediated by forskolin, we asked whether an agent that blocks release of calcium from IP_3 receptor-mediated intracellular stores would affect forskolin-induced potentiation. In Fig. 8, forskolin in the presence of 100 μM TMB-8, did not potentiate the EPSP slope. After 60 min washout of forskolin, a significant depression was noted (86 ± 4%, n = 7, P < 0.05, paired t-test). It appeared that intracellular calcium might also be modulating basal neurotransmission, as there was a noticeable decrease in the EPSP slope after 10 min perfusion with TMB-8 alone.

Effect of IP3 receptor blockade on forskolin-stimulated CREB and ERK phosphorylation - Results of the Western blot experiments displayed in Fig. 9 show that 100 μM TMB-8 had no effect on basal CREB or ERK phosphorylation when perfused for 20 min. When forskolin was subsequently applied in the continued presence of TMB-8, CREB phosphorylation was

significantly depressed when compared with forskolin alone (88 ± 9%, n = 3, $P < 0.05$, ANOVA). In contrast, TMB-8 had no effect on forskolin-stimulated increases in phosphorylation of ERK-1 and ERK-2 (ERK-1, 173 ± 33%, n = 3; ERK-2, 136 ± 14%, n = 3, $P > 0.05$, ANOVA). Thus, intracellular release of calcium via IP_3 receptors contributes to the ability of forskolin to mediate CREB phosphorylation, while having no effect on ERK activation.

DISCUSSION

Understanding how memory is encoded by the brain requires an examination of the molecular/cellular events set into motion when neurons are activated. Potentiation of the EPSP observed after bath application of forskolin is one model for increased activity in the hippocampus. This increase does not require electrical stimulation (Voulalas and Sarvey, 1997b). Furthermore, increased neurotransmitter release evoked by forskolin has been reported in field CA1 (Chavez-Noriega and Stevens, 1994). Although the exact mechanism whereby elevation of cAMP induces a potentiation is unknown, one can begin to address the question of the role of cAMP in synaptic plasticity with this model.

The principal findings of this study were the following. First, not only is a protein known to be responsive to increased amounts of intracellular cAMP activated by forskolin (CREB), but proteins (ERK-1 and 2) thought previously to be primarily responsive to calcium and growth factor receptor stimulation are also activated by forskolin in the hippocampal dentate gyrus. Second, blocking the ability of MEK to activate ERK-1 and 2 also renders the ERKs incapable of being activated by forskolin. Third, we have observed that basal CREB phosphorylation is modulated by NMDA receptors and IP_3 receptors in such a manner that it also is unable to be activated by forskolin. Finally, ERK activation is independent of some tyrosine kinase activities while CREB

phosphorylation appears to depend on activation of multiple pathways, including those involving as yet undefined tyrosine kinases.

The activation of ERK-1 and 2 by forskolin in the dentate gyrus is intriguing, given the limited amount of evidence for cAMP-dependent stimulation of these MAPKs. Until recently, the mechanism of MAPK activation was believed to proceed primarily through activation of Ras and Raf-1, either by elevation of intracellular calcium or by stimulation of growth factor receptors (for review, see Campbell et al., 1995). Interestingly, recent work describes a Ras- and calcium-independent pathway leading to activation of ERK-1 and 2. In PC12 cells, cAMP activates the small G protein Rap1 to stimulate B-Raf, which itself leads to ERK activation via MEK (Vossler et al., 1997). Since Rap1 can also inhibit Raf-1 in a cAMP-dependent manner, the overall effect of cAMP on ERK activation in a particular cell will be reflective of the expression of either Raf-1 or B-Raf in that cell, or the balance between the effect of each Raf isoform in cells expressing both proteins.

The inability of forskolin to stimulate the ERKs in the presence of the MEK inhibitor PD098059 strengthens the idea that cAMP's effect on the ERKs is upstream of MEK. Inhibition of forskolin-stimulated CREB phosphorylation by PD098059 implies that ERK-1 and/or 2 activation is necessary for phosphorylation of CREB. The optimal conditions for CREB activation appear to be complex, though, considering that two

broad spectrum tyrosine kinase inhibitors block CREB phosphorylation without affecting ERK activation. In this regard, the disparity between the effects of lavendustin A versus MDC suggests that stimulation of parallel pathways is required for the full activation of CREB.

Another critical molecule regulating CREB phosphorylation is calcium. In this study, we noted that not only is the NMDA receptor required for forskolin-induced CREB activation, but it also modulates basal CREB phosphorylation. Interference with activation of IP_3 receptors, in contrast, did not affect basal CREB phosphorylation, but did prevent forskolin-mediated increases. We explored the contribution of IP_3 receptors to forskolin-mediated increases in CREB phosphorylation based on a study in which we found that TMB-8 produced a concentration-dependent decrease in potentiation (Voulalas and Sarvey, 1997b). In the current study, we found a complete blockade of potentiation with 100 μM TMB-8, which yielded a slight depression after 60 min of wash.

In summary, the regulation of CREB and ERK phosphorylation by global elevation of cAMP appears to be complex. Our data illustrate that in the dentate gyrus, cAMP can lead to the activation of CREB, ERK-1 and ERK-2. Although basal and stimulated phosphorylation of these molecules is modulated by different molecules, cross-talk in signaling occurs at the level of MEK. Further dissection of the pathways leading to activation of CREB and ERK in these neurons may in the future

be facilitated by activity-specific antibodies targeting upstream pathways and modulators.

FIG. 1. **Stimulation of adenylyl cyclase induces potentiation in the dentate gyrus.** Slices treated with 50 μM forskolin (*closed circles*) but not the inactive forskolin analog, 50 μM dideoxyforskolin (*open circles*) exhibit a long-lasting increase in the initial slope of the EPSP. Bath application of forskolin or dideoxyforskolin for 20 min is indicated by the bar. Each point represents the mean ± SEM.

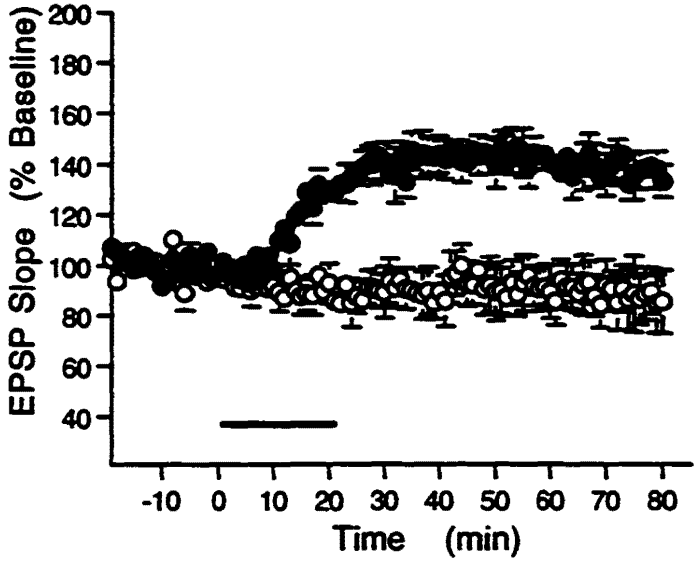
EPSP Slope (% Baseline)
200
180
160
140
120
100
80
60
40
-10
0
10
20
30
40
50
60
70
80
Time (min)

Fig. 1

FIG. 2. **Time course of activation of CREB by forskolin in the dentate gyrus.** Slices were removed at the times indicated. Dentate extract (10 μg protein per lane) was separated by SDS-PAGE and the Western blot was probed with an anti-phosphoCREB antibody. *A*, slices show CREB phosphorylation peaking at about 20 min after start of perfusion with 50 μM forskolin (antibody source, M.E. Greenberg). *B*, 50 μM dideoxyforskolin does not increase CREB phosphorylation (antibody source, New England Biolabs).

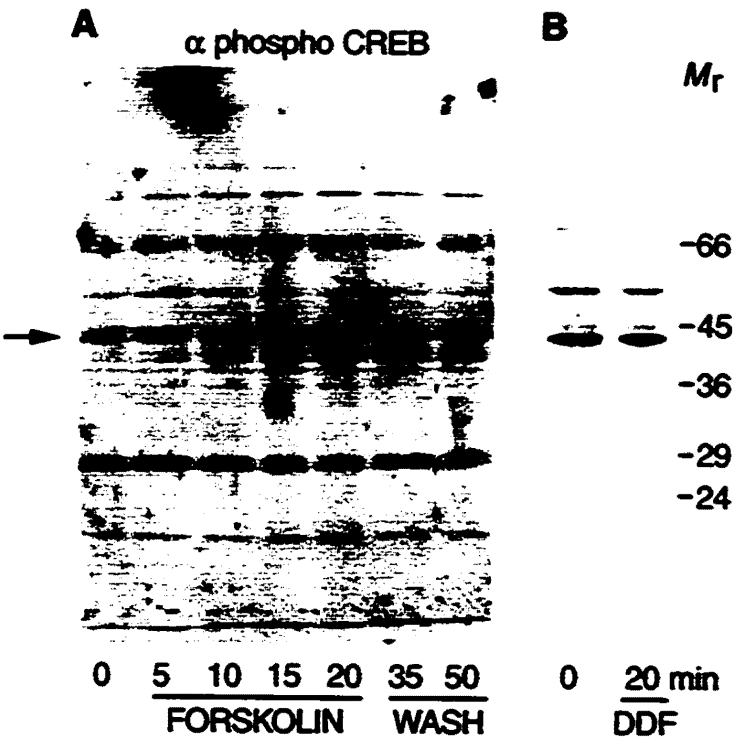
A
α phospho CREB
B
M_r
-66
-45
-36
-29
-24
0 5 10 15 20 35 50
FORSKOLIN WASH
0 20 min
DDF

Fig. 2

FIG. 3. **Time course of activation of p42 and p44 MAPK by forskolin in the dentate gyrus.** Slices were removed at the times indicated. Dentate extract (10 μg protein per lane) was separated by SDS-PAGE and the Western blot was probed with an anti-phosphoMAPK antibody. *A*, slices show MAPK phosphorylation peaking at about 15 min after start of perfusion with 50 μM forskolin (antibody source, M.E. Greenberg). *B*, 50 μM dideoxyforskolin does not increase MAPK phosphorylation (antibody source, Promega). *C*, Reprobing blots in *A* and *B* with anti-MAPK demonstrates equal loading across lanes.

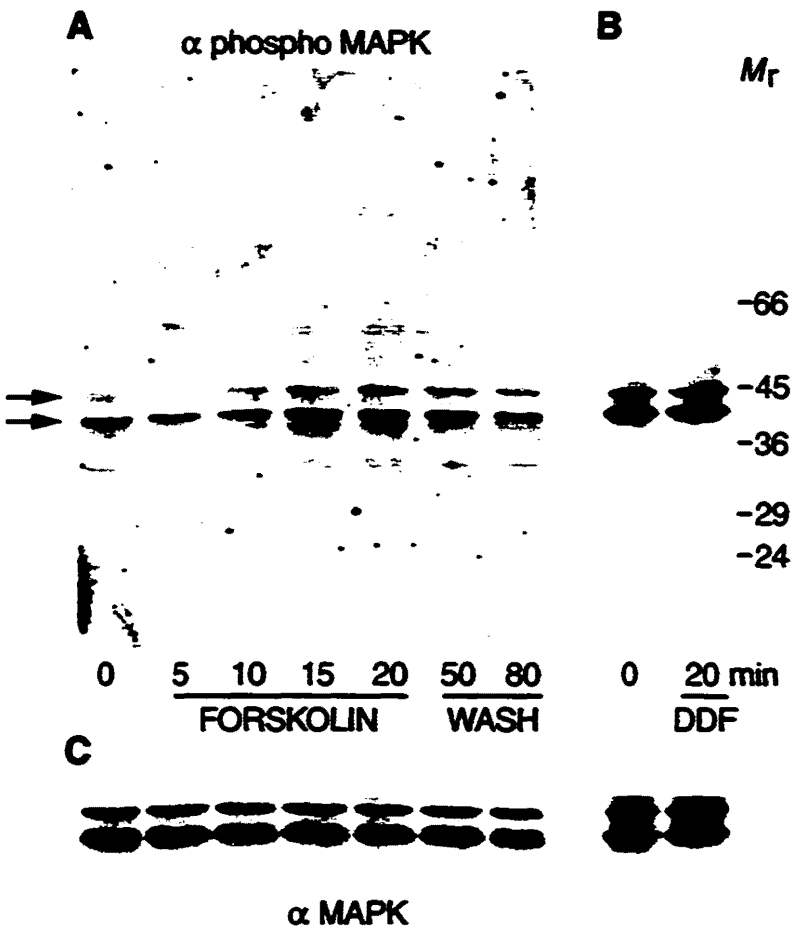
A
α phospho MAPK
B
M_r
-66
-45
-36
-29
-24
0 5 10 15 20 50 80
FORSKOLIN
WASH
0 20 min
DDF
C
α MAPK

Fig. 3

FIG. 4. **Effect of two tyrosine kinase inhibitors and a MEK inhibitor on forskolin potentiation.** Slices were treated with lavendustin A (5 μM, open circles), the erbstatin analog MDC (20 μM, closed squares), or the MAPK kinase inhibitor PD098059 (50 μM, open squares) for 20 (lavendustin A, MDC) or 30 min (PD098059) prior to, during, and for 20 min after perfusion with 50 μM forskolin. MDC and PD098059, but not lavendustin A, cause a significant reduction of potentiation 60 min after washout of forskolin. Closed circles show time course of response to perfusion with forskolin alone (50 μM). Each point represents the mean ± SEM.

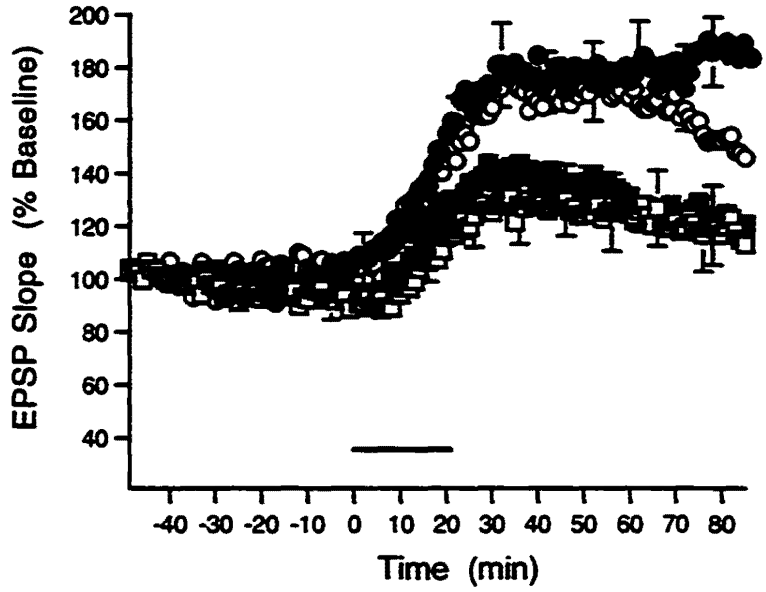
EPSP Slope (% Baseline)
200
180
160
140
120
100
80
60
40
-40 -30 -20 -10 0 10 20 30 40 50 60 70 80
Time (min)

Fig. 4

FIG. 5. **Effect of two tyrosine kinase inhibitors and a MEK inhibitor on MAPK phosphorylation.** Time "0" was tissue that had been equilibrated in ACSF for at least three hours, and was pharmacologically naive. "I" indicates tissue that had been exposed to either 5 μM lavendustin A (20 min), 20 μM MDC (20 min) or 50 μM PD098059 (30 min). Lanes labeled "FORSK + I" had been pretreated with lavendustin A, MDC or PD098059, then perfused with forskolin for 20 min, while maintaining the inhibitor in solution. Dentate extract (10 μg protein per lane) was probed with an anti-phosphoMAPK antibody (Promega). Neither lavendustin A (5 μM) nor MDC (20 μM) block forskolin-mediated increases in MAPK phosphorylation. PD098059 (50 μM) reduces basal MAPK phosphorylation and blocks forskolin-stimulated increases in MAPK phosphorylation. Reprobing blots with anti-MAPK antibody (Santa Cruz) demonstrates that increases in phosphorylated MAPK are not due to increases in total protein loaded in the respective lane.

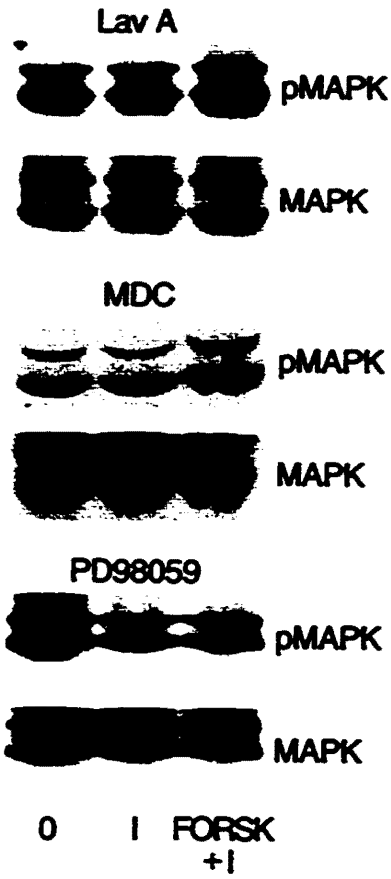

Fig. 5

FIG. 6. **Effect of two tyrosine kinase inhibitors and a MEK inhibitor on CREB phosphorylation.** Slices were removed before treatment (0), after pre-treatment with the stated kinase inhibitor (I) and at the end of 50 μM forskolin application (FORSK + I). Dentate extract (10 μg protein per lane) was separated by SDS-PAGE and the Western blot was probed with an anti-phosphoCREB antibody (NEB). Lavendustin A (5 μM), MDC (20 μM) and PD098059 (50 μM) all block forskolin-mediated increases in CREB phosphorylation.

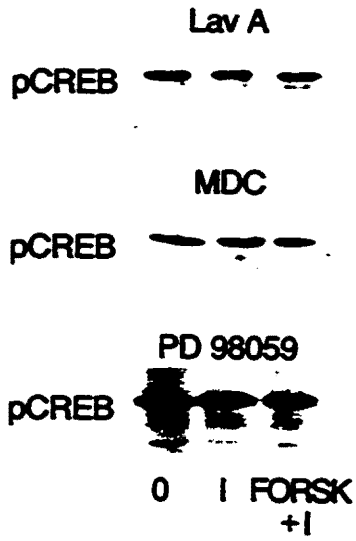

Fig. 6

FIG. 7. **Effect of NMDA receptor blockade on CREB and MAPK phosphorylation.** Slices were removed before treatment (0), after pre-treatment with 20 μM D-APV and at the end of 50 μM forskolin application (FORSK + APV). *A*, Blockade of NMDA receptors with D-APV decreases basal CREB phosphorylation and blocks increases mediated by forskolin. B, NMDA receptor blockade does not affect basal or forskolin-stimulated increases in MAPK phosphorylation. Reprobing blots with anti-MAPK antibody demonstrates that increases in phosphorylated MAPK are not due to increases in total protein loaded in the respective lane.

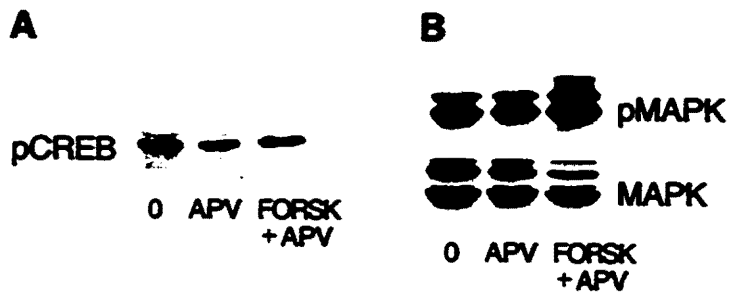

Fig. 7

FIG. 8. **Effect of IP_3 receptor blockade on forskolin potentiation.** Slices were treated with 100 μM TMB-8 for 20 min prior to, during and for 20 min after washout of 50 μM forskolin (open circles). TMB-8 interferes with the IP_3-mediated release of calcium from internal stores and blocks forskolin-induced potentiation. Data from Fig. 1 shows the typical time course of forskolin potentiation (open squares). Each point represents the mean ± SEM.

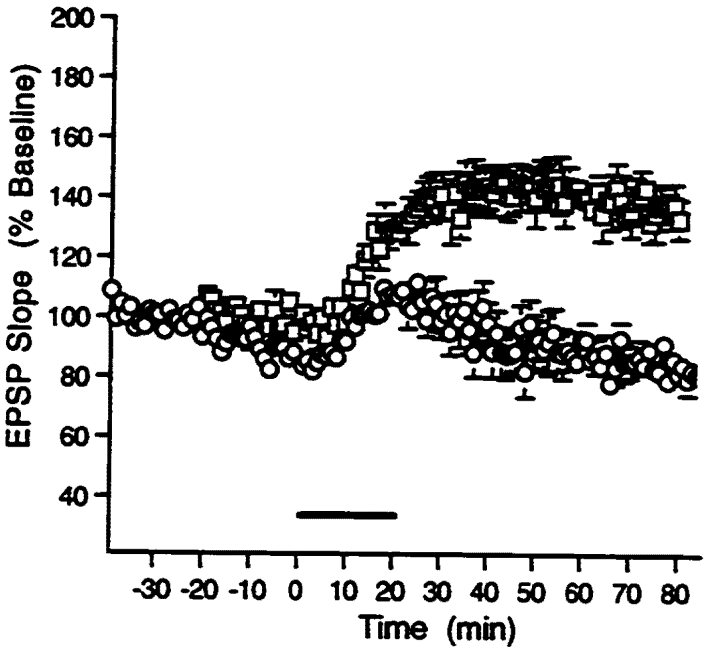
EPSP Slope (% Baseline)
200
180
160
140
120
100
80
60
40
-30 -20 -10 0 10 20 30 40 50 60 70 80
Time (min)

Fig. 8

FIG. 9. **Effect of IP_3 receptor blockade on CREB and MAPK phosphorylation.** Dentate minislices were removed from the chamber before TMB-8 application ("0"), 20 min after 100 μM TMB-8 application ("TMB") and 20 min after forskolin and TMB-8 were applied together in the bathing medium ("FORSK + TMB"). *A*, Blockade of IP_3 receptors with TMB-8 blocks increases in CREB phosphorylation mediated by forskolin. *B*, IP_3 receptor blockade does not affect basal or forskolin-stimulated increases in MAPK phosphorylation. The blot reprobed with anti-MAPK antibody demonstrates that increases in phosphorylated MAPK are not due to increases in total protein loaded in the respective lane.

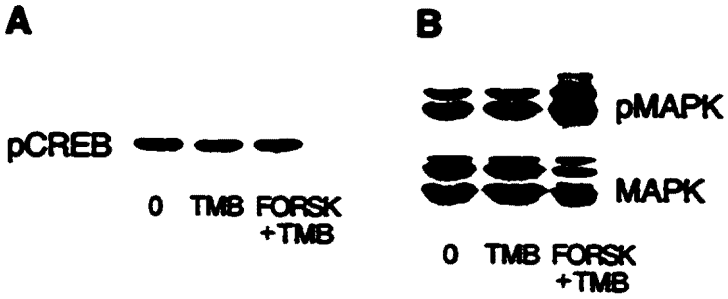

Fig. 9

Sequential activation of CREB and MAP kinase by LTP in the dentate gyrus

Summary

CREB and the mitogen-activated protein kinases (MAPKs) are transducers of membrane-to-nucleus signaling that are activated by phosphorylation (Gonzalez and Montminy, 1989; Dash et al., 1991; Sheng et al., 1991). LTP, a model for learning and memory (Bliss and Lømo, 1973; Sarvey, 1988; Collingridge and Bliss, 1995), is blocked by protein synthesis inhibitors (Stanton and Sarvey, 1984, 1987; Deadwyler et al., 1986; Matthies, 1989; Otani and Abraham, 1989; Nguyen et al., 1994; Frey and Morris, 1997) as is acquisition of memory (Rosenzweig, 1984). Studies using cell culture models have revealed elevations in phosphorylated CREB with LTP-inducing stimuli (Deisseroth et al., 1996; Sheng et al., 1990); in field CA1 of hippocampal slices, MAPK phosphorylation increases following high-frequency stimulation (English and Sweatt, 1996). We induced LTP in dentate gyrus of urethane-anesthetized rats and in dentate gyrus minislices to map the time course of CREB and MAPK phosphorylation after induction of LTP. LTP-inducing stimulation *in vivo* and *in vitro* resulted in an increase in CREB phosphorylation that was NMDA receptor-dependent. This occurred subsequent to increases in phosphorylation of the MAPKs, ERK-1 and ERK-2, which were NMDA receptor-independent *in vivo* and *in vitro*. LTP *in vitro* was blocked by the specific MAPK kinase inhibitor PD098059. These data demonstrate sequential and differential activation of CREB

and MAPKs by LTP induction, and suggest a link between NMDA receptor activation and CREB phosphorylation. They also demonstrate a requirement for MAPK activation for maintenance of LTP.

Results and Discussion

The time course of LTP expression *in vivo* is shown in Fig. 1a. A robust increase in population spike amplitude reached 320 ± 30% while the EPSP slope increased to 126 ± 4% ($P < 0.05$, $n = 9$, paired t-test) was observed 60 min after high-frequency stimulation (HFS) of perforant path fibers. Fig. 1b shows that the bulk of phosphorylated CREB detected with an anti-phosphoCREB antibody specifically recognized Ser-133-phosphorylated CREB found in the nuclear fraction of dentate extracts (Ginty et al., 1993). To facilitate protein quantitations for Western blot analyses, we routinely worked with whole cell extracts of isolated dentate gyrus. The anti-phosphoCREB antibody reacted primarily with a doublet of approximately 43 and 44kD. We have found that the higher molecular weight band in these extracts co-migrated with an immunoreactive protein found in the cytosolic fraction and was not modulated by activity.

CREB phosphorylation increased rapidly and remained elevated after delivery of HFS (Fig. 1c). The animals given low frequency stimulation (LFS) had undergone surgery and test stimulation (0.033Hz) of perforant path fibers, but no HFS. The level of CREB phosphorylation in the LFS sample and 5 min following HFS was detectable, but low. By 10 min after induction of LTP the levels of phosphoCREB had risen dramatically; phosphorylation peaked by 20 min (242 ± 53% of LFS, $n = 5$). This maximal level was maintained through 60 min

after stimulation, but fell back to baseline levels within 2 hours. Note that phosphorylation of a cross-reactive 37kD protein as well as the 44kD protein were not influenced by LTP induction and did not change over time. The time course of CREB phosphorylation also reflects the sensitivity of LTP to protein synthesis inhibitors within the first hour after HFS (Stanton and Sarvey, 1984; Frey and Morris, 1997).

The NMDA receptor antagonist MK-801 blocks LTP in the anesthetized rat (Abraham and Mason, 1988). As demonstrated in Fig. 1d, there was no potentiation 5 min (136 ± 17%, n = 6) or 20 min (96 ± 28%, n = 3) after HFS ($P < 0.05$, paired t-test). Tissue samples were collected at these time points for Western blot analysis. Two animals designated as LFS were injected with MK-801 and sacrificed after 30 min of low frequency test stimulation, without delivery of HFS. The other two were sacrificed after HFS, at times when CREB phosphorylation would have been maximal (see Fig. 1c). In the presence of MK-801, HFS did not lead to an increase in CREB phosphorylation (86 ± 20%, n = 4; $P > 0.05$, paired t-test), as shown in Fig. 1e. The requirement for NMDA receptors to activate CREB is consistent with a role for CREB in NMDA receptor-dependent LTP. Other studies utilizing cultured hippocampal neurons found CREB phosphorylation to be NMDA receptor-dependent (Ginty et al., 1993; Deisseroth et al., 1996).

Similar results were obtained from *in vitro* hippocampal slices. Fig. 2a shows the time course of LTP induced in the medial perforant path of the dentate gyrus. The

initial slope of the EPSP was increased to 160 ± 17% of baseline values 60 min after HFS (n = 5, P < 0.05, paired t-test). This paradigm induces LTP in the dentate gyrus that lasts more than three hours (see Fig. 4). In Fig. 2b a representative time course analysis of CREB phosphorylation in minislices of dentate gyrus is shown. The sample taken at 0 min was unstimulated; other samples were collected 30s, 1, 5, 10, 20 and 30 min after delivery of HFS. Phosphorylation of the 43kD protein increases 5 to 10 min after HFS, peaks between 10 and 20 min (167 ± 18%, n = 3) and returns to basal levels 30 min following induction of LTP. This time course follows LTP-induced cAMP increases in the dentate which peak in less than 5 min (Stanton and Sarvey, 1985c). The observation that CREB phosphorylation had a delayed onset and was sustained longer *in vivo* than *in vitro* could be explained either by different stimulus paradigms used *in vivo* versus *in vitro*, modulatory influences of intact connections *in vivo*, or the use of anesthetic *in vivo*.

The role of NMDA receptors in modulating CREB phosphorylation after HFS in slices is demonstrated in Fig. 2c. These slices exhibited a low basal level of CREB activation. In lane 2, perfusion of slices with 20μM D-APV for 30 min led to a decrease in the basal level of phosphoCREB. The presence of D-APV during delivery of HFS prevented increases in phosphoCREB from reaching levels noted in the absence of D-APV (117 ± 16%, n = 3, P < 0.05, paired t-test). Thus, blockade of NMDA receptors reduced the increase in

phosphorylation of CREB seen after HFS *in vitro* just as it did *in vivo*.

We and others have noted that there is a basal level of CREB phosphorylation that is sensitive to NMDA receptor blockade (Ginty et al., 1994). Here we demonstrate that perfusion of slices for 30 min with 20 μM D-APV produced a characteristic decrease in basal CREB phosphorylation (Fig. 2d). This effect is specific for the active isoform of APV, as perfusion of slices for 30 min with the inactive isomer L-APV had no effect on basal CREB phosphorylation (Fig. 2d). Incubation of slices for 30 min in ACSF containing 10mM EGTA and no calcium also led to a decrease in the amount of phosphoCREB present in these slices. This result supports the idea that NMDA receptors mediate an essential influx of extracellular calcium to maintain basal CREB phosphorylation for transcriptional activity. Electrophysiological evidence exists for tonic activity of NMDA receptors in hippocampal slices (Sah et al., 1989).

The anti-Active MAPK antibody detected both p44 ERK-1 and p42 ERK-2 in dentate extracts. The basal level of ERK-2 detected was consistently higher than that of ERK-1. Following induction of LTP in vivo by HFS, phosphorylation of both ERK-1 and ERK-2 increased rapidly and transiently, peaking in less than 10 min (ERK-1, 584 ± 177%, n = 3; ERK-2, 363 ± 123%, n = 3) and falling to baseline levels again within 20 min (Fig. 3a).

In contrast to induction of CREB phosphorylation by HFS, phosphorylation of ERK-1 and 2 *in vivo* was not blocked by the NMDA receptor antagonist MK-801 (ERK-1, 349 ± 19%, $n = 3$; ERK-2, 317 ± 61%, $n = 3$; $P < 0.05$ for both, paired t-test) as shown in Fig. 3b. Blots in Fig. 3a and 3b were reprobed with an anti-ERK-1 antibody that recognized both ERK-1 and 2, and the reactivity observed indicated nearly equal total ERK protein loaded across all lanes.

The time course of MAPK phosphorylation after LTP induction was also examined in dentate minislices. As seen in Fig. 3c, HFS resulted in an increase in both ERK-1 and 2 phosphorylation within 1 min (ERK-1, 326 ± 135%, $n = 5$; ERK-2, 278 ± 60%, $n = 6$) that was sustained for less than 20 min. This characteristic time course was nearly identical to the pattern of MAPK activation noted *in vivo*. The peak of activation occurring within 5 to 10 min correlates well with the translocation of MAPK to the nucleus. Despite some cytoplasmic pooling, a significant amount of phosphorylated MAPK is nuclear within 5 min (Chen et al., 1992; Fukuda et al., 1996; Whitmarsh and Davis, 1996). Another study conducted in field CA1 found that LTP induction stimulated only ERK-2 phosphorylation (English and Sweatt, 1996). This distinction of utilization of ERK-1 and ERK-2 in dentate versus CA1 is supported by the localization of ERK-1 mRNA in the dentate granule cells, but not CA1 pyramidal cells in adult rats (Thomas and Hunt, 1993).

Pretreatment of slices with 20μM D-APV for 30 min prior to HFS did not prevent increases in phosphorylation of ERK-1 and 2 (ERK-1, 552 ± 154%, n = 3; ERK-2, 179 ± 29%, n = 3; $P > 0.05$ for both, Student's t-test) demonstrated in Fig. 3d. Therefore, as *in vivo*, NMDA receptor activity was not required for phosphorylation of these MAPKs in the dentate gyrus. Our results have supported the idea that increases in ERK phosphorylation are not NMDA receptor dependent, as neither APV *in vitro* nor MK-801 *in vivo* blocked HFS-induced increases. Such results indicate that neuronal activity not involving NMDA receptors may induce MAPK phosphorylation. In contrast, another group found that increases in ERK-2 phosphorylation in field CA1 were NMDA receptor dependent (English and Sweatt, 1996). The difference in outcome between labs could be reflective of differential utilization of the ERKs in dentate versus CA1. In a related study, we have found that cAMP-dependent potentiation in the dentate gyrus activates CREB in an NMDA receptor-dependent manner, while MAPK activation was NMDA receptor-independent (Voulalas and Sarvey, 1996).

Given that increases in MAPK activity appeared to correlate strongly with induction of LTP, we assessed the requirement for MAPK in the expression of LTP. Recently, PD098059 has been described and characterized as a specific inhibitor of the kinase activating MAPK (MAPK kinase, or MEK) (Dudley et al., 1995). In Fig. 4a, slices perfused with PD098059 for 30 min demonstrate a decrease in basal levels of

activated MAPK. We treated slices with 50μM PD098059 for 30 min prior to induction of LTP, and continued perfusion of the drug for 30 min following delivery of the high-frequency train. As seen in Fig. 4b, the stimulus paradigm utilized leads to the expression of a long-lasting potentiation that is induced without blockade of $GABA_A$ receptors with bicuculline. This potentiation demonstrates a more short-lived nature when the MEK inhibitor PD098059 is present in the bath, resulting in a return of the potentiated responses to baseline values 90 min after LTP has been induced. This observation in conjunction with the NMDA receptor-independent activation of MAPK implies that MAPK activation is necessary, but not sufficient for the expression of LTP in the dentate gyrus. In area CA1, PD098059 has also been proven to block NMDA receptor-dependent LTP (English and Sweatt, 1997). In the cited study, two induction paradigms were used; one which produced a "modest" amount of potentiation and a second, which produced a more robust potentiation of the initial slope of the EPSP. Induction of LTP in CA1 using these two paradigms appears to be controversial, in terms of its dependency on NMDA receptors (Impey et al., 1996). Thus, the finding by English and Sweatt that MAPK phosphorylation is NMDA receptor-dependent contrasts with our findings in the dentate gyrus. This may reflect regional differences or differences in stimulus paradigms.

Using the *in vivo* preparation as well as the *in vitro* preparation and two different stimulus paradigms, this study

has confirmed the idea that LTP induction is directly correlated with activation of MAPK and CREB by phosphorylation in a complex system, the intact hippocampus. Demonstration of blockade of a long-lasting potentiation in the dentate gyrus by PD098059 provides direct evidence for a role for MAPK in the maintenance of LTP. Although a causal link between CREB phosphorylation and LTP induction remains to be made, the temporal relationship between these events suggest that CREB may be important for eliciting changes in gene expression required for the expression of LTP.

Methods

***In vivo* preparation.** Male Sprague-Dawley rats (200 to 350g) were anesthetized with urethane and placed in a stereotaxic head holder. Burr holes were drilled to accommodate electrodes placed at the following coordinates relative to Bregma: A-P 7.9mm, M-L 4.2mm to stimulate the medial perforant path, and A-P 3.9mm, M-L 2.3mm to record in the dentate gyrus. Bipolar, monophasic stimulus pulses (200μs duration) were delivered to the perforant path, while a recording electrode was lowered into the dentate gyrus until the maximal positive excitatory postsynaptic potential (EPSP) was recorded. Test stimuli of low frequency (LFS, 0.033 Hz) were implemented to isolate responses and monitor potentials during baseline periods. LTP was induced with high frequency stimulation (HFS) consisting of four trains of eight pulses at 400Hz, with a 10s interval between trains; current intensity was set to evoke a two-thirds maximal population spike (200-350μA, 200μs). When used, (+)MK-801 (1mg/kg i.p.) was injected at least 3 hours prior to delivery of the HFS. At designated time points after HFS, the animals were decapitated, the hippocampus rapidly removed and the dentate gyrus isolated and frozen in liquid nitrogen. Samples were stored at -80°C until processed.

***In vitro* preparation.** Male Sprague Dawley rats (100-200g) were anesthetized with ketamine hydrochloride (100mg/kg i.p.) and decapitated. Hippocampal slices (400μm) were prepared as

described elsewhere (Burgard and Sarvey, 1991). Slices were placed in an interface chamber at 32-34°C and perfused at 3ml/min with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 1.75, KH_2PO_4 1.25, $MgSO_4$ 1.3, $NaHCO_3$ 26, $CaCl_2$ 2.4, and dextrose 10. ACSF was bubbled with 95% CO_2/5% O_2 to achieve a pH of 7.2 to 7.4. Medial perforant path fibers were stimulated and extracellular field potentials were obtained using glass micropipettes filled with 2M NaCl. Test stimuli were delivered every 30s. LTP was induced using a train of 100Hz, 2s; current intensity ranged from 150 to 250μA and pulse width from 50-70μs. This typically evoked a population spike that was 50 to 60% of the maximal response. Dentate minislices used for Western blot analyses were prepared as previously described (Voulalas and Sarvey, 1997c). Minislices were generated from a single group of 2 - 3 animals for each time course experiment, and were all incubated simultaneously in the same chamber to minimize variation between preparations. HFS was delivered to both the supra- and infrapyramidal blades of the dentate gyrus; current intensity and pulse width for HFS were selected based on the input-output curves of representative slices in each preparation. In experiments where D-APV (20μM in 20μM NaOH) and PD098059 (50μM in 0.1% DMSO) were used, slices were perfused for 30-40 min in the antagonist prior to delivery of HFS. DMSO (0.1%) had no effect on basal transmission (data not shown). At designated times after stimulation, minislices

were rapidly frozen in liquid nitrogen and stored at -80°C to await processing.

Statistical analysis of drug effects was carried out in Statview using paired t-tests. A probability of 0.05 was selected as the level of statistical significance. All data are expressed as the mean ± SEM.

Preparation of dentate gyrus extracts. Twenty to 50mg of dentate gyrus tissue (one dentate gyrus or approximately 15 minislices) were homogenized in 10mM Tris, pH 7.4 containing aprotinin, leupeptin, pepstatin, sodium orthovanadate, disodium molybdate, glycerol-2-phosphate, benzamidine, sodium fluoride and PMSF to suppress protease and phosphatase activity. Protein content was quantified using the BCA Protein Assay Kit (Pierce) to insure equivalent loading. Equal loading was confirmed by Ponceau S staining of Western blots.

Western blot analyses. Dentate gyrus whole cell extracts (10µg) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were blocked with 10% BSA/TBS-Tween, and antibody incubations were done in 1% BSA/TBS-Tween. Immunoreactive proteins were visualized using an HRP-conjugated secondary antibody followed by enhanced chemiluminescence (ECL, Amersham). Quantitation of Western blots was carried out using Quantity One (PDI). Statistical analysis of drug effects on CREB and MAPK phosphorylation was carried out in Statview using paired t-tests. A probability of 0.05 was selected as the level of statistical

significance. All data are expressed as the mean ± SEM. Anti-Active MAPK antibody used for *in vivo* analyses was purchased from Promega and anti-ERK-1 antibody was purchased from Santa Cruz. The anti-phosphoCREB and anti-phosphoMAPK antibodies were a generous gift from M.E. Greenberg.

Acknowledgments. We thank D. Kirkby for *in vivo* training; M. Moore for expert technical assistance and advice on the manuscript; and T. Curran and D. Ginty for helpful discussions. These studies were supported by the National Institutes of Health and the Uniformed Services University of the Health Sciences.

Figure 1 LTP increases CREB phosphorylation *in vivo*. **a**, Time course of LTP. Population spike amplitude is designated by closed circles, EPSP slope by open circles. Inset shows representative sweeps one minute prior to and 2h after HFS. **b**, Phosphorylated CREB is detected in whole cell (1) and nuclear (3), but not cytosolic (2) extracts of dentate gyrus. **c**, CREB phosphorylation peaks 10 to 20 min after HFS and is maintained through 60 min. **d**, MK-801 (1mg/kg) blocks LTP of the population spike and EPSP. **e**, MK-801 blocks HFS-induced increases in CREB phosphorylation. Western blots show results from four animals.

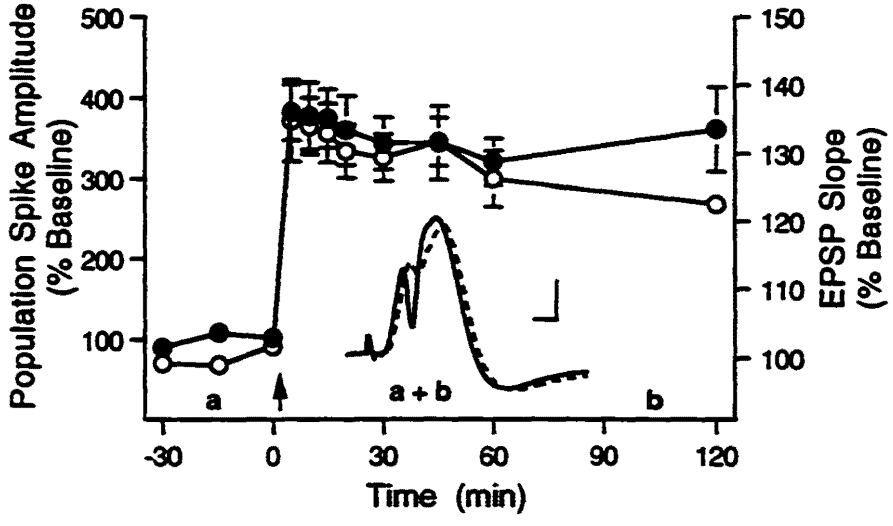
Population Spike Amplitude
(% Baseline)
500
400
300
200
100
150
140
130
120
110
100
EPSP Slope
(% Baseline)
a
a + b
b
-30
0
30
60
90
120
Time (min)

Fig. 1a

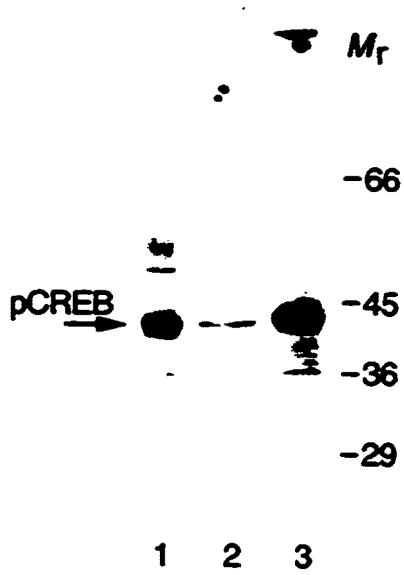

Fig. 1b

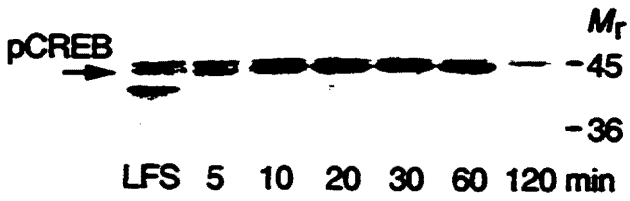

Fig. 1c

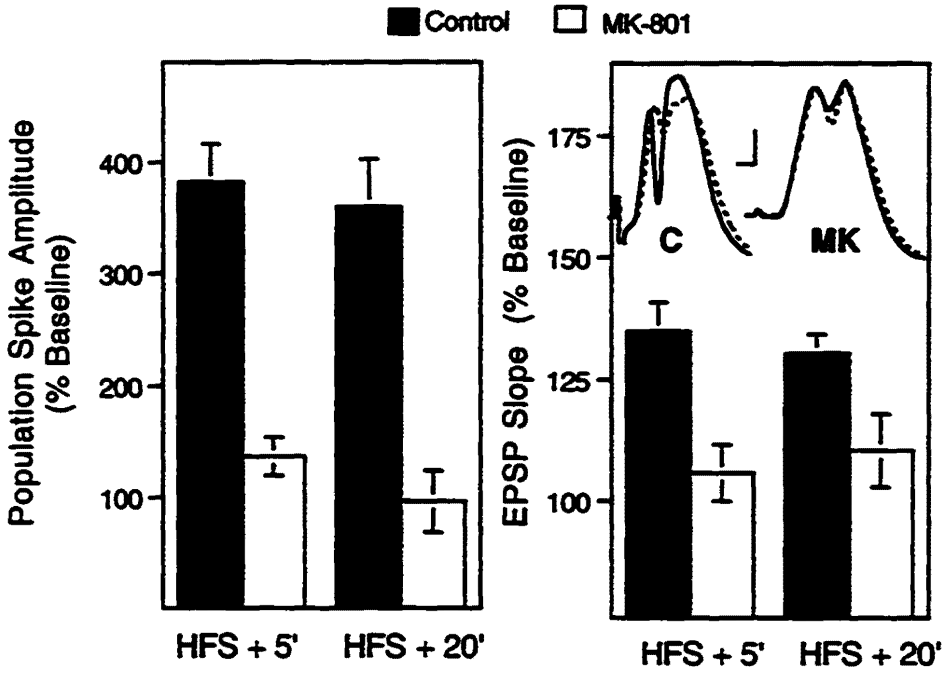
Control
MK-801
Population Spike Amplitude (% Baseline)
400
300
200
100
HFS + 5'
HFS + 20'
EPSP Slope (% Baseline)
175
150
125
100
C
MK
HFS + 5'
HFS + 20'

Fig. 1d

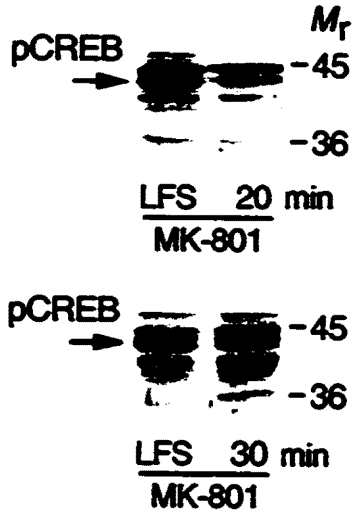

Fig. 1e

Figure 2 LTP *in vitro* increases NMDA receptor-dependent CREB phosphorylation. **a**, Time course of LTP following delivery of HFS. Inset shows representative sweeps one minute prior to and 1h after HFS. **b**, LTP was induced in minislices and samples were removed at the times indicated. **c**, D-APV reduces HFS-induced increases in CREB phosphorylation. **d**, Extracellular calcium modulates basal CREB phosphorylation. Control tissue (0) was collected prior to 30 min incubation of minislices with either D-APV, L-APV or ACSF with 10mM EGTA and no calcium. Equivalent loading of protein in each lane is exemplified by a constant amount of a 37kD and a 44kD immunoreactive protein.

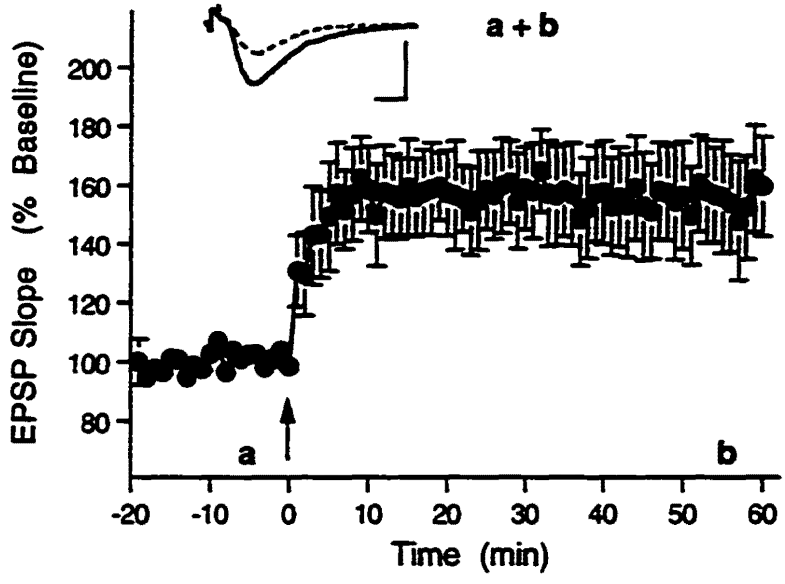
a + b
EPSP Slope (% Baseline)
200
180
160
140
120
100
80
a
b
-20
-10
0
10
20
30
40
50
60
Time (min)

Fig. 2A

Fig. 2b+c

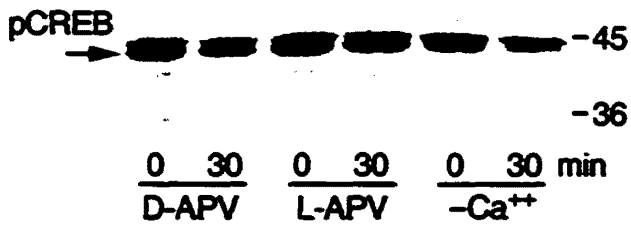

Fig. 2d

Figure 3 LTP-inducing stimuli activate MAPK. **a**, MAPK activation *in vivo* peaks 5 min after delivery of HFS and returns to baseline levels within 20 min. **b**, MK-801 does not block HFS-induced increases in MAPK phosphorylation. **c**, LTP was induced in minislices, and samples were removed at the times indicated. Western blots in **3c** and **3d** were probed with an anti-phosphoMAPK antibody obtained from M.E. Greenberg. **d**, D-APV does not block HFS-induced increases in MAPK phosphorylation.

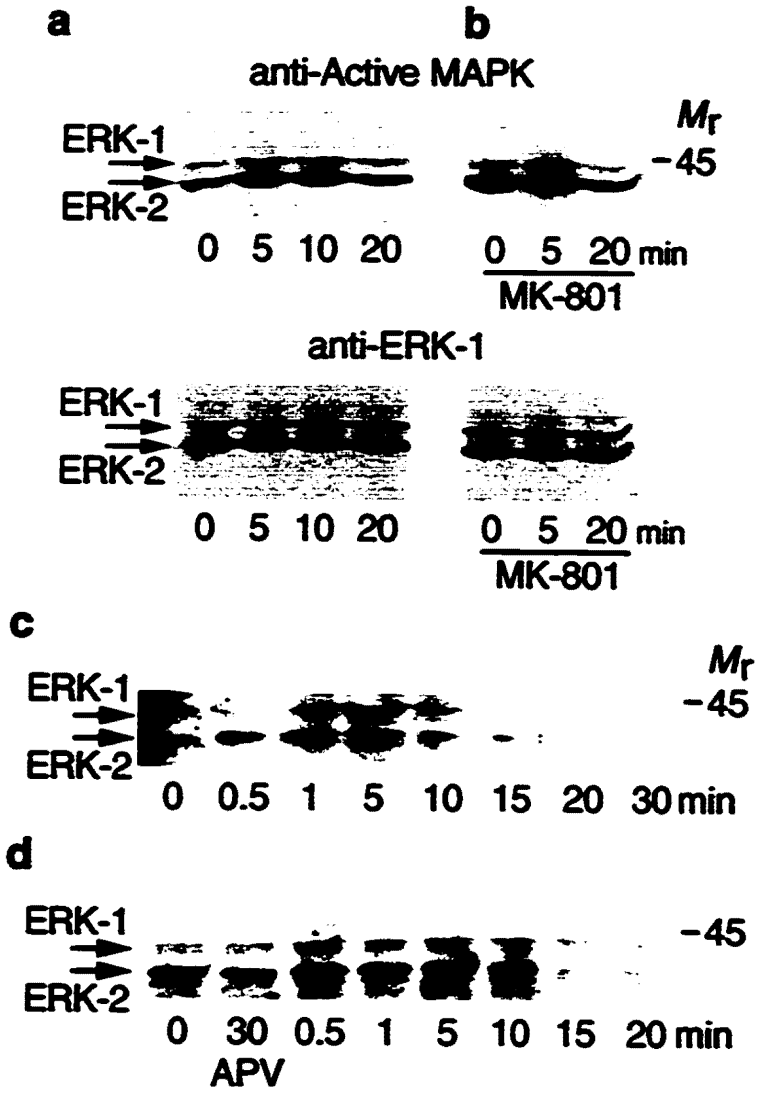
a
b
anti-Active MAPK
M_r
ERK-1
ERK-2
-45
0 5 10 20
0 5 20 min
MK-801
anti-ERK-1
ERK-1
ERK-2
0 5 10 20
0 5 20 min
MK-801
c
M_r
ERK-1
ERK-2
-45
0 0.5 1 5 10 15 20 30 min
d
ERK-1
ERK-2
-45
0 30 0.5 1 5 10 15 20 min
APV

Fig. 3

Figure 4 Inhibition of MAPK kinase (MEK) reduces MAPK phosphorylation and long-term expression of LTP. **a**, Untreated slices demonstrate a basal level of ERK-1 and ERK-2 phosphorylation that is reduced with a 30 min exposure to PD098059 (50 μM). **b**, PD098059 abbreviates long-lasting increase in EPSP slope by HFS. Control slices are designated by closed circles, PD098059-treated slices by open circles.

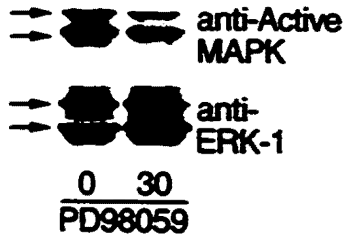

Fig. 4a

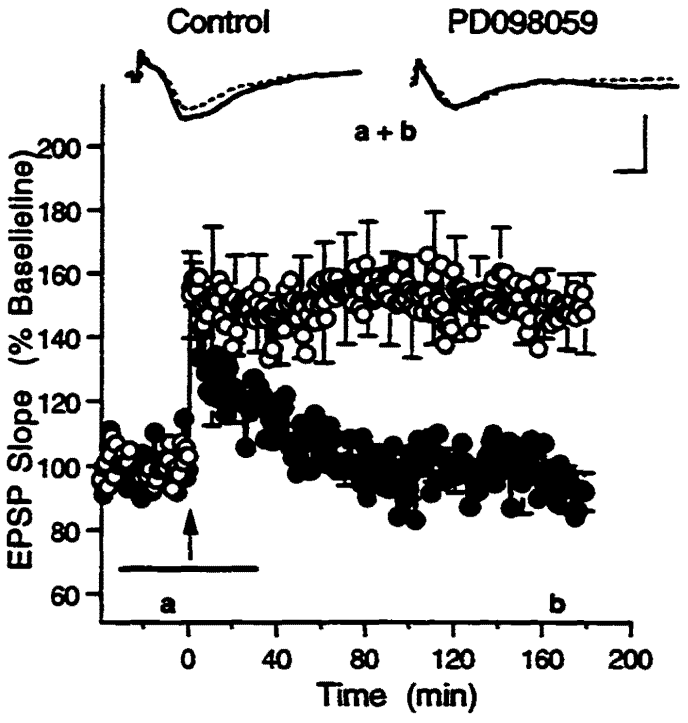
Control
PD098059
a + b
EPSP Slope (% Baselleline)
200
180
160
140
120
100
80
60
a
b
0
40
80
120
160
200
Time (min)

Fig. 4b

DISCUSSION

My ambition in this body of work was to deepen our understanding of hippocampal synaptic plasticity by providing a molecular definition of the phenomenon. LTP is the premier cellular model for learning and memory in mammals. Unfortunately, one problem with the model is the lack of a precise description of the stimulus paradigm required for induction of the phenomenon. My goal was to establish an induction protocol that would reflect the cellular mechanisms required for transducing a short-term stimulus into a long-term change in neuronal activity.

The discovery that the NMDA receptor acted as a gate in the expression of LTP in the hippocampus provided a first step toward achieving such a goal (Collingridge et al., 1983). In our laboratory, another form of long-lasting potentiation had been described in the dentate gyrus that was induced by brief bath-application of norepinephrine (Stanton and Sarvey, 1985a). This was referred to as norepinephrine-induced long-lasting potentiation, or NELLP. The induction of NELLP is dependent upon activation of β-adrenergic receptors and NMDA receptors for its expression. Our laboratory made the interesting finding that LTP in the dentate gyrus was dependent on β-adrenergic receptor activation. This interdependence of NMDA receptors and β-

adrenergic receptors provided important information about plasticity in the dentate gyrus, and was critical for the development of the hypothesis put forth in this thesis.

Concurrent with the initiation of this thesis project, the finding was made that LTP in field CA1 was blocked by tyrosine kinase inhibitors (O'Dell et al., 1991). If this sensitivity of LTP to tyrosine kinase inhibitors was confirmed in the dentate gyrus, it could provide a link between the NMDA receptor- and β-adrenergic receptor-dependency for LTP induction. Beginning with what was known about LTP and NELLP, a hypothesis was developed. NMDA receptor activation provides a mechanism for increasing intracellular calcium. β-Adrenergic receptors activate adenylyl cyclase to generate cAMP. In neither LTP nor NELLP was there an obvious link to tyrosine kinase activity. Therefore, comparison of an NMDA receptor-dependent versus a β-adrenergic receptor-dependent form of potentiation could be carried out, in the context of its dependence on tyrosine kinase activity. My original hypothesis was that the calcium-dependent and the cAMP-dependent potentiation converged at some point downstream from activation of the membrane-bound receptors, with a substrate for tyrosine phosphorylation residing at that point of convergence.

The method of choice for inducing a cAMP-dependent form of potentiation was bath application of forskolin to

dentate slices. Forskolin was preferred over the cAMP analogs dibutyryl-cAMP and 8-bromo-cAMP because these agents have a direct effect on adenosine receptors and give rise to a depression of synaptic transmission followed by a potentiation. Although other investigators have used forskolin to study cAMP-dependent potentiation in the hippocampal formation, no one had examined in detail its mechanism of action. In this portion of the thesis project, I first determined that forskolin potentiation was activity-independent, since electrical stimulation was not required for its expression. The ability to induce potentiation soley by bath application of a drug provided a technical advantage for biochemical examination of stimulated slices. Electrical stimulation of a large number of slices to generate a time course of events is extremely cumbersome, and becomes more complicated as one pharmacologically characterizes the biochemical effects of electrically stimulated slices.

I also determined that the source of calcium was not critical for induction and maintenance of this potentiation. This study was the first to provide evidence for forskolin potentiation being NMDA receptor-independent. Amongst all of the published reports on the subject of forskolin potentiation, not one addressed a mechanism for the induction of the potentiation. Typically, dideoxyforskolin was used to demonstrate the lack of a specific effect seen with forskolin. Another novel observation in this body of work

was that IP_3 receptors contribute to forskolin potentiation. IP_3 receptors mediate calcium release from an intracellular store. TMB-8 has been identified as a drug that interferes with the calcium release mechanism activated by IP_3 receptors. The dramatic effect of TMB-8 on basal transmission in the dentate gyrus at concentrations approaching 100 μM was not expected, but may reflect the importance of the IP_3 receptor-sensitive intracellular store in maintaining a calcium equilibrium in the resting state. Nevertheless, the role of IP_3 receptors in synaptic plasticity needs to be investigated further. Finally, the pharmacological characterization regarding the contribution of dantrolene-sensitive and IP_3 receptor-sensitive calcium stores provides insight into the ways in which a cell can achieve the levels of calcium necessary for long-lasting changes in activity.

Attempts to determine the molecular mechanisms underlying LTP in the hippocampus have been met with technical difficulties inherent in both the slice preparation and the intact animal. *In vivo*, stimulation of perforant path fibers at a point where the fibers anatomically converge (the angular bundle) is believed to induce LTP throughout most of the dentate gyrus. In contrast, stimulation of perforant path fibers in a discrete region of the dentate gyrus in slices is thought to induce LTP in a smaller percentage of cells, with the occurrence decreasing with

distance from the stimulating electrode. In addition, the connectivity in the intact dentate gyrus versus the isolated dentate gyrus could itself give rise to a distinct pattern of biochemical events. Therefore, in addition to the *in vitro* slice preparation, I conducted a number of experiments using the *in vivo* anesthetized animal preparation. Early in the course of the project, I determined that physically isolating the dentate gyrus from the rest of the hippocampus was desirable. This would in theory improve the signal-to-noise ratio in the coordinated biochemical analyses, and also provide a first step toward distinguishing between events occurring in the dentate gyrus versus CA1 or CA3. With the *in vivo* preparation, *post-mortem* isolation of the dentate gyrus following LTP induction was quite easy, as it required only three additional steps following decapitation of the animal and dissection of the hippocampus. The *in vitro* slice preparation presented the slightly more difficult problem of isolating the dentate at some point during the experiment. A preliminary approach involved isolation of the dentate from the rest of the hippocampus during the initial dissection, but this preparation never yielded electrophysiologically healthy tissue. Another protocol was devised whereby a micro-scalpel was used to cut out the dentate area once hippocampal slices had been cut and placed into the chamber, but cutting away so much tissue was very difficult to do with tissue that did not readily adhere to a surface for trimming. Finally, a method was developed in which the dentate gyrus

was isolated in such a way as to yield electrophysiologically robust slices. Although other investigators report the use of minislices for biochemical analyses, there is no published demonstration of electrophysiological responses or LTP from a minislice.

As outlined above, the comparison of calcium-dependent versus cAMP-dependent potentiation was to be based on the ability of both to activate a downstream target that was a substrate for tyrosine kinases. The first target I selected was MAPK, which is activated by calcium as well as growth factor receptors, and is a substrate for phosphorylation of both tyrosine and threonine by the dual-specificity protein kinase MEK (Ghosh et al., 1994; Campbell et al., 1995; Whitmarsh and Davis, 1996). The advent of anti-phosphoMAPK antibodies facilitated the examination of phosphorylation of this molecule, by eliminating the need to add large quantities of ^{32}P-ATP to the slice incubation chamber. Finally, a second relevant target selected for this study was CREB. When this thesis project was initiated, CREB was known to be activated by cAMP and calcium, through protein kinase A (PKA) and calcium-calmodulin kinase II (CaMKII), respectively. Within the last two years, data has been published demonstrating CREB phosphorylation by pp90^{RSKII}, which itself is a substrate for MAPK phosphorylation. Within the last three months, more evidence has been published

describing a mechanism for MAPK activation via cAMP (Vossler et al., 1997).

The role of the MAPKs and the transcription factor CREB in plasticity has been examined primarily in non-neuronal cell cultures (e.g., PC12 cells), in cultured hippocampal pyramidal cells and in area CA1 of hippocampal slices. Given our lab's experience with LTP in dentate slices, and the fact that *in vivo* recordings are technically easier to carry out in the dentate gyrus than in CA1, I chose to examine the ability of potentiating stimuli to induce phosphorylation of CREB and the MAPKs in the dentate gyrus. In addition, CREB and MAPK phosphorylation in LTP *in vivo* had not been examined. In the second phase of this thesis project, I proceeded to determine whether chemical induction of potentiation by forskolin *in vitro*, and induction of LTP *in vivo* and *in vitro* would lead to activation of CREB and the MAPKs by phosphorylation. For these experiments, time course analyses were carried out in order to map the temporal pattern of activation of MAPK and CREB following induction of potentiation. The information derived from these experiments not only provided the confirmation that MAPK and CREB were being phosphorylated in parallel with both LTP induction and cAMP-mediated potentiation, but that activation of MAPK preceded activation of CREB. This pattern was most striking with LTP *in vivo* and *in vitro*, and lends support to studies initially carried out in single cell preparations suggesting

that MAPK may in certain conditions be activating CREB through $pp90^{RSKII}$. Experimental results demonstrating that the MEK inhibitor PD098059 blocks LTP provides an additional link between MAPK and LTP. The future demonstration that LTP-induced increases in MAPK and CREB are also blocked by the MEK inhibitor would provide conclusive evidence of the link between LTP, NMDA receptors, MAPK and CREB. These relationships in LTP are at the moment merely hypothesized based on cell culture models.

Finally, one must determine whether a change in activity of a protein is truly related to the expression of potentiation, and not just an epiphenomenon of neuronal activity per se. Therefore, I investigated the sensitivity of changes in MAPK and CREB phosphorylation to agents that are known to interfere with the expression of LTP and forskolin potentiation. The finding that LTP-induced CREB phosphorylation, but not MAPK phosphorylation was blocked by NMDA receptor antagonists in the dentate gyrus *in vivo* and *in vitro* may indicate that CREB phosphorylation is a specific indicator of LTP-related cellular processes, whereas MAPK phosphorylation may be reflective only of an increase in neuronal activity. The assumption would therefore be that MAPK activation primes the system for LTP, but other cellular events occurring in parallel are required. For example, the sensitivity of forskolin-potentiation and CREB phosphorylation to a tyrosine kinase inhibitor and an IP_3

receptor inhibitor provide strong support for other pathways whose activity is required for full expression of potentiation. Of greatest interest is the ability of an NMDA receptor antagonist to consistently decrease basal CREB phosphorylation. A limited number of published reports have suggested tonic activation of NMDA receptors, but the demonstration of a biochemical effect of an NMDA receptor antagonist on the neuronal population in a slice provides greater support for such an idea.

To conclude, the model in Fig. 1 of the discussion is a first attempt to outline a series of events requiring calcium and cAMP for the expression of potentiation in the dentate gyrus. In this model, the recently described mechanism for signal descrimination by cAMP is via the small G protein Rap-1. This protein is activated by cAMP (presumably through phosphorylation by PKA) to perform the following two functions. It can stimulate B-Raf to activate MEK and MAPK, or it can inhibit Raf-1 to inhibit MEK and MAPK. Stimulation of the MAPK cascade in the dentate gyrus by cAMP implies that B-Raf is the isoform active in these neurons. A scenario could also exist where both Raf-1 and B-Raf are present in the same neurons, but the final outcome, in terms of MAPK activation, depends upon other factors modulating the balance between inhibition of Raf-1 and activation of B-Raf by cAMP. At this time, the question

remains open, awaiting the experimental demonstration of either Raf-1 or B-Raf, or both, in the dentate gyrus.

To summarize, a time course of activation of MAPK and CREB has been established in the dentate gyrus such that any stimulus paradigm purported to induce a long-lasting change in synaptic efficacy should be observed in a rapid induction of MAPK activity followed by an increase in activated CREB. The presumption is that together, these two molecules are initiating increases in transcriptional activity that are essential for the change in synaptic weight believed to underlie the long-lasting structural changes in connectivity in the brain when a new memory is formed.

Figure 1. Proposed model of mechanism in the hippocampal dentate gyrus whereby calcium and cAMP can lead to the activation of MAPK and CREB by phosphorylation.

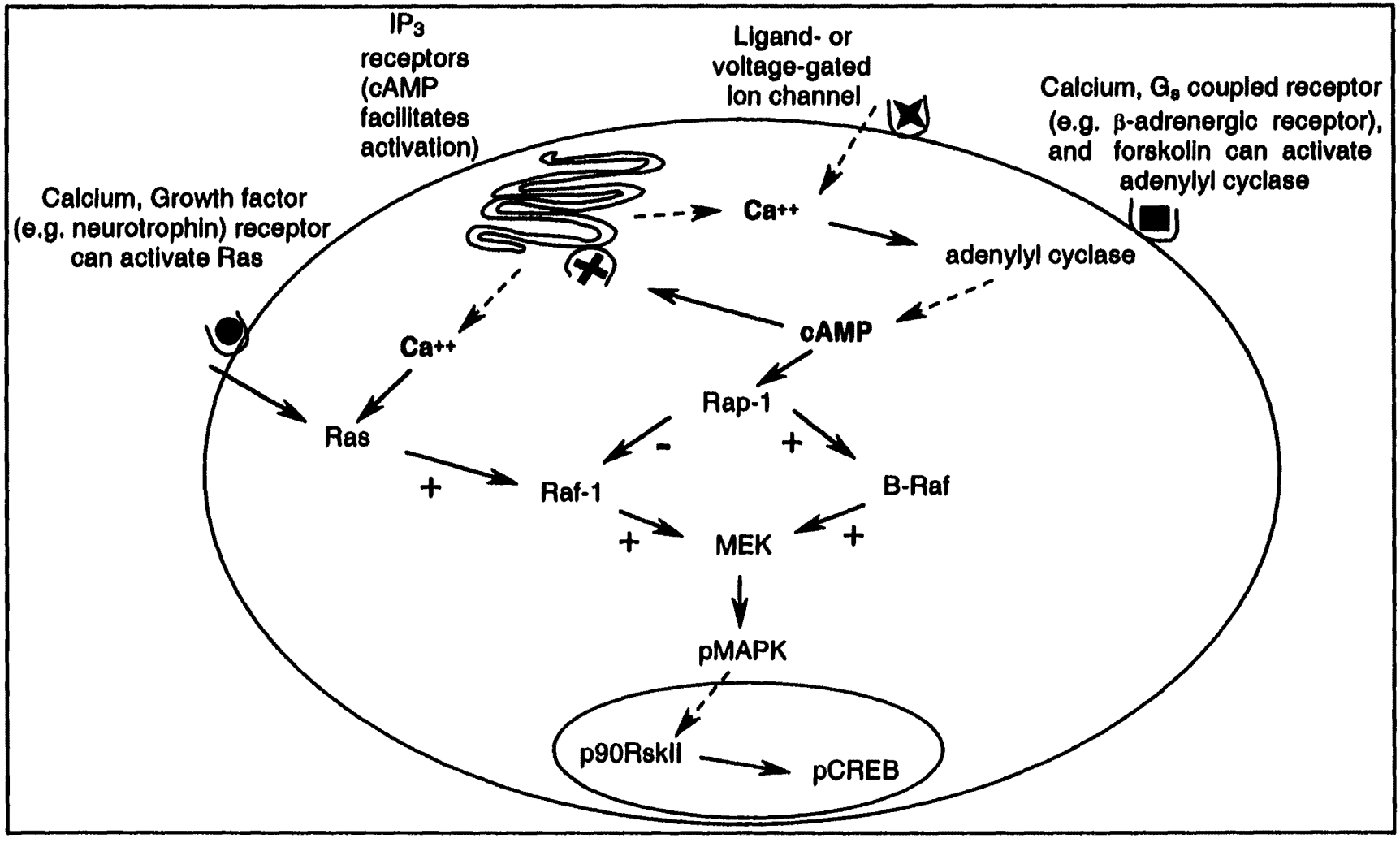
IP3 receptors (cAMP facilitates activation)
Ligand- or voltage-gated ion channel
Calcium, Gs coupled receptor (e.g. β-adrenergic receptor), and forskolin can activate adenylyl cyclase
Calcium, Growth factor (e.g. neurotrophin) receptor can activate Ras
Ca++
adenylyl cyclase
Ca++
cAMP
Rap-1
Ras
-
+
+
Raf-1
B-Raf
+
+
MEK
pMAPK
p90RskII
pCREB

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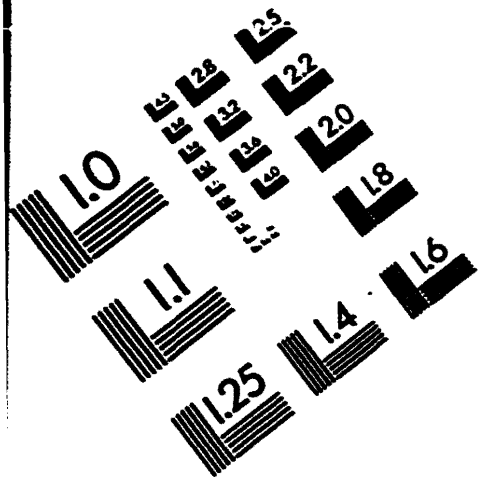
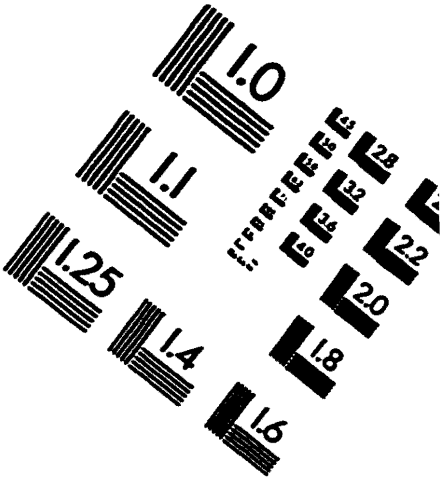
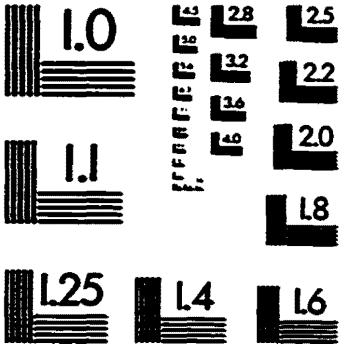
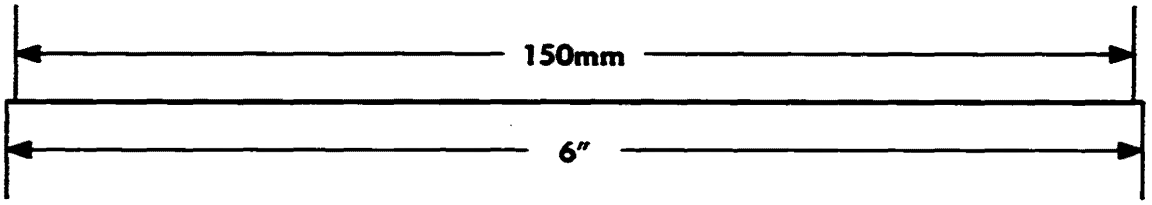
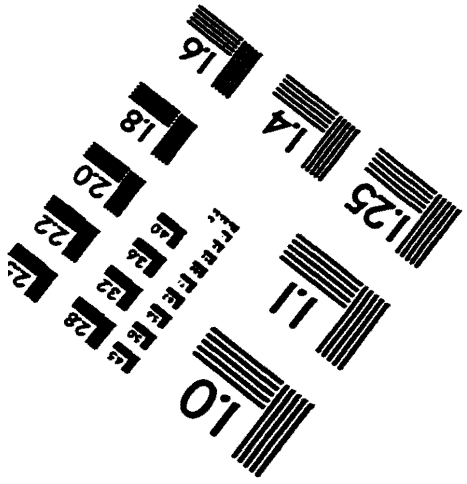
IMAGE EVALUATION
TEST TARGET (QA-3)











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